AGROBACTERIUM-MEDIATED TRANSFORMATION OF RED CLOVER AND TOBACCO WITH THE PEANUT STUNT VIRUS COAT PROTEIN GENE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1995

ACKNOWLEDGMENTS

In accomplishing my academic goals at the University of Florida's Department of Agronomy, I am particularly appreciative for the generosity and financial support provided by the National Council for Scientific Development (CNPq - Brazil), and the Institute for Agricultural Research of the State of Santa Catarina (EPAGRI - Brazil).

I would also like to express my gratitude to Dr. Kenneth H. Quesenberry, who provided the opportunity for this experience through his contribution of ideas and advice, and through his personal involvement in my work. Additionally, I wish to extend my appreciation to the other members of the committee, Drs. Paul Pfahler, Rex Smith, Gloria Moore and David Wofford, for their continuous support during this research. Dr. Pfahler was particularly involved in the project through his friendship and the many ideas shared during the discussion of the problems encountered in this research; he also helped me with editing this dissertation. Dr. Wofford taught me a great deal on plant breeding, particularly on diallel crosses. Special gratitude is expressed to Drs. Gloria Moore and Rex Smith, who offered their research laboratories, enabling me to carry out the most critical experiments in bacteriology and molecular biology, and who provided constant advice in these areas.

Dr. Francis Zettler kindly allowed the use of his laboratory for the immunological aspects of this project.

Special thanks go to Jeffery Seib, Mark Elliot, and Reggie Salazar, Senior Biologists in Agronomy, Plant Pathology and Horticulture Departments, respectively, who provided extensive assistance in elucidating the procedures required for this work in the areas of molecular biology, microbiology and virology. Their patience with my limitations on these subjects was essential to the successful outcome of these procedures.

Personal gratitude goes to Maria Joao Pereira, who offered her friendship during my studies here in Florida. Diane Luth, Yuyu Poerba and Denis Beliaev helped me with ideas. Loan Ngo, David Moon and Amy Branum helped me in many experimental procedures. Thank you.

Mens sana in corpore sano. My gratitude goes to Team "A" at the Infirmary, and principally to Drucilla Vallerio, who took care of my physical health in a loving way, and to Wayne Bottom, Wayne Griffith and Linda Lewis, who greatly helped me to keep my mental and spiritual health in good shape.

My family and friends back in Brazil always kept strong the ties of love and care between us. Thanks them all.

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December 1995

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Major Department: Agronomy

Peanut stunt cucumovirus (PSV) causes serious disease in many crops, including red clover (*Trifolium pratense* L.) and tobacco (*Nicotiana tabacum* L.). A possible method of control is coat protein-mediated protection. We report results on transformation of red clover and tobacco with the plasmid pMPSV4-43, which contains the PSV coat protein (CP) gene. We also report results on the effects of medium pH and acetosyringone (AS) on the growth and virulence of *A. tumefaciens*, and on the efficacy of kanamycin and geneticin in growth inhibition of non-transformed red clover plants.

Fifty $\mu g\ ml^{-1}$ kanamycin proved to be sufficient to hinder the development of red clover. Geneticin at doses as low as 3 $\mu g\ ml^{-1}$ provided a suitable alternative, particularly since this antibiotic was resistant to

degradation by heat during autoclaving, which was not observed with kanamycin.

The addition of AS to the medium, coupled with acidic conditions, were more conducive to plant transformation by *A. tumefaciens*. These conditions negatively affected the initial growth of the bacteria, although 72 hours after inoculation the effects were no longer apparent. Medium acidity had negative effects on callus formation in red clover, but the effects were counterbalanced by the increase in virulence verified in acidic medium.

Transformation of red clover explants with *A. tumefaciens* Z707(pMPSV4-43) and EHA101(pMPSV4-43), and of tobacco explants with EHA101(pMPSV4-43), was performed, and a number of plants regenerated on medium containing kanamycin. Gene integration was assayed through polymerase chain reaction using primers for the *npt*II and *psv-cp* genes. Gene expression was assayed through enzyme-linked immunosorbent assay using antibodies against the NPTII protein. A number of tobacco and red clover plants showed positive results for both ELISA and PCR assays.

CHAPTER 1
INTRODUCTION

The refinements in molecular techniques for plant improvement have made possible the incorporation, into plants, of genes from widely different species, including other plant species, animal species, bacteria and viruses. These techniques aim primarily at overcoming the barriers that preclude the transfer of genes by natural means.

The progress in obtaining transgenic plants in the last few years, after the initial successes reported (De Block et al., 1984; Horsch et al., 1984), has been fantastic. A vast array of species has now been transformed; resistance to viruses, insects and diseases has already been incorporated, using foreign genes (Delannay et al., 1989; Fishhoff et al., 1987).

The most challenging questions posed to scientists dealing with gene transfer are: (i) what are the appropriate genes to transfer; (ii) how can those genes be obtained; and (iii) how can the genes be transferred from donor to acceptor (Grumet et al., 1987). A further question involves the conditions for the expression of the transferred genes, once integrated into the plant genome.

The introduction of genes for resistance to viruses in plants has received much attention lately. Cloned viral genes have been used, as opposed to host genes. This approach takes advantage of the fact that in any host-pathogen interaction there are certain pathogen-encoded functions that are essential to the pathogen, but not to the host. The goal is to cause disruption of one of these functions.

Coat protein-mediated resistance is perhaps the most common method in genetic engineering for virus resistance. Other approaches include the use of satellite sequences, antisense RNAs, and the whole genome of a mild virus. Whereas all methods have proven to be effective in a range of situations, the use of coat protein genes seems to be the most promising approach.

Red clover (*Trifolium pratense* L.) is an important forage legume, widely grown in many parts of the world. It provides good quality forage, either for fresh consumption or for hay or silage production. One of the problems that the crop presents in the United States is infection by various plant viruses, including peanut stunt virus (PSV), which causes systemic mottle in the plants. Tobacco (*Nicotiana tabacum* L.) is also infected by this virus.

This project aimed at creating red clover and tobacco plants that expressed the peanut stunt virus coat protein gene (*psv-cp*). The approach selected was the creation of

transgenic plants containing the *psv-cp* gene through *Agrobacterium tumefaciens*-mediated gene transfer. In summary, the steps involved were (i) the co-cultivation of red clover and tobacco explants with *A. tumefaciens*; (ii) the selection of putative transgenic plants containing the transferred gene; and (iii) the assessment of the presence of the gene in the recovered plants.

A. tumefaciens strain Z707, harboring the binary vector pMPSV4-43, was initially used in transformation of red clover. *A. tumefaciens* strain EHA101 was transformed with the plasmid pMPSV4-43, and used for further transformation of red clover and tobacco.

In addition, we studied two other aspects of plant transformation, namely: (i) the effects of antibiotic type and concentrations in the regeneration medium on the efficiency of selection of transformed plants; and (ii) the effects of acetosyringone, a phenolic compound, on the growth and virulence of *A. tumefaciens*.

CHAPTER 2
LITERATURE REVIEW

Plant Genetic Transformation

Many techniques for gene transfer in plants have been attempted, with varying degrees of success. Within these, *Agrobacterium*-mediated gene transfer, DNA microinjection, microbombardment, and gene transfer into protoplasts are the most promising. I shall briefly deal with each method, and then present details of genetic engineering of plant virus resistance using *A. tumefaciens* as the vector. In addition, the importance of peanut stunt virus, as well as biological aspects of this organism, will also be covered. The use of the phenolic compound acetosyringone as a virulence-inducer in *Agrobacterium* will be discussed later, followed by a review on the use of kanamycin and geneticin as selectable antibiotics in experiments dealing with plant transformation, when the *npt*II gene is part of the transformation vector.

Microinjection

In this technique, microcapillaries and microscopic devices are used to deliver DNA directly inside cells without destroying them, allowing regeneration. This is a

commom method in animal transformation, particularly for the creation of transgenic mice; Jaenish and Mintz (1974) reported the first genetically-transformed mice derived from blastocytes injected with simian virus 40 DNA. However, in plants the method is much more difficult, because of the presence of cell walls and of many detrimental enzymes in the vacuoles (Songstad et al. (1995). This method has been applied to microspore-derived proembryos and to zygotic proembryos of cereals, although in these cases, no molecular proof of integrative transformation was obtained when the progenies of the transformed plants were tested (Neuhaus et al., 1987).

A successful attempt was made in 1987, using oilseed rape microspore-derived embryoids (Neuhaus et al., 1987). The transformation efficiency was high (from 30 to 50%), but the the authors described the process as time-consuming, since the delivery of genetic material has to be done in one cell at a time.

Biolistics or Microprojectile Bombardment-Mediated Transformation

Biolistics is an abbreviation for biological ballistics. This method can be described as the introduction of substances into intact cells and tissues using high-speed microprojectiles (Sanford, 1992). As in microinjection, the main achievement of this process is to overcome the barrier imposed by the cell wall.

High-density metals such as tungsten and gold are utilized as microprojectiles. These are covered with the DNA to be inserted. The method has been found to be effective in a wide range of plants, including both dicotyledonous and monocotyledonous (see Sanford, 1992 for a comprehensive review). The biolistic technique is becoming widely used, in part because it does not require a wound response from the plant, which is necessary for *Agrobacterium*-mediated systems.

Klein et al. (1987) pioneered the field of microprojectile transformation, using a conventional gun powder cartridge and tungsten microprojectiles. Improvements in this system replaced the conventional gunpowder with high-pressure helium, an innovation that was commercially exploited by DuPont (Russell-Kikkert, 1993). The use of an airgun was also adopted, presenting the advantage of low cost over previous systems. Oard et al. (1990) and Oard (1993) described the use of this system and its application for transient gene expression in wheat, maize and rice cells. Yet another improvement was the particle inflow gun, or PIG, in which solenoid valves are used to direct helium inflow. This system was initially described by Finer et al. (1992) and Vain et al. (1993a). Transient and stable maize transformations using this method have been described by Vain et al. (1993b).

A less traditional approach for the use of the microprojectile bombardment was adopted by Bidney et al. (1992); sunflower apices were bombarded with particles without any DNA, as a means of providing uniform wounding in the plant tissues, before transformation was done using the *Agrobacterium*-mediated approach.

A successful application of the biollistic technique for the incorporation of viral coat protein in rice is given by Qu et al. (1992). Transformation for viral protection has also been reported by Marsh et al. (1993) and Murry et al. (1993). Various other transient or permanent transformation in plants from the *Poaceae* family have also been reported; for a review, see Songstad et al., (1995).

Microprojectile bombardment presents the unique advantage over other methods of being able to deliver DNA into cells of any tissue of any organism, and is applicable to species that are recalcitrant to transformation via *Agrobacterium* or through protoplast culture (Songstad et al., 1995).

Plant Protoplast Transformation

Protoplasts can be defined as plant cells in which the cell wall has been eliminated, usually by enzymatic means. It had already been shown that plant viruses and viral nucleic acids could be taken up by protoplasts (Takebe, 1975), and this leads to the extension of the method to

recombinant DNA. Takebe et al. (1971) described strategies for plant regeneration from tobacco mesophyll protoplasts. *Poaceae* members proved to be more difficult to be successfuly cultured (Vasil, 1987), but some successes have already been obtained.

Direct uptake of recombinant DNA is aided by various treatments of the cells, including the use of chaotropic ions, polymers (e.g. polyethylene glycol, polyvinyl pyrrolidone), calcium shock, and electroporation, the application of brief pulses of electrical potential (Goodman, 1990). In addition, plant protoplasts from different species can be fused to produce hybrids. The interest in this technique is related to the prospect that wider crosses than are possible by sexual means can be achieved by protoplast fusion (Dodds and Roberts, 1982). Crossway et al. (1986) microinjected *Agrobacterium* plasmid DNA into tobacco protoplasts with a regeneration frequency of 14% and 6%, respectively for nuclei and cytoplasm injections.

Agrobacterium-Mediated DNA Transfer

The bacterial genus *Agrobacterium* is well-known for its remarkable and unique capacity to transfer DNA into plant cells. The transferred DNA (T-DNA) is part of a large plasmid, the tumor-inducing plasmid (pTi). The T-DNA is integrated into the plant DNA, and subsequent expression of

its genes leads to the formation of tumors (Otten et al., 1992).

The transfer during infection is due to the activity of the plant-inducible virulence genes *vir*, located on the Ti plasmid, and of the chromosomal genes *chv* (Douglas et al., 1985; Hooykass, 1989). The T-DNA regions are flanked by border sequences that define the region to be transferred to the infected plant cell. The T-DNA contains 8 to 13 genes, including some for the production of phytohormones, responsible for the formation of the tumors when transferred into plant cells. These are not preserved in constructs used for the production of transgenic plants, thereby eliminating the ability of the bacteria to induce tumors (Binns, 1990; Gasser and Fraley, 1989).

The DNA transfer events have been reviewed by Goodman (1990), and can be summarized as follows. Several genes, both from the plasmid and from the bacterial chromosome, are activated in response to chemical signals elicited from the plant by wounding. At least three chromosomal loci are involved in the attachment of bacteria to plant cells. Various kinds of single and double-stranded (ss and ds, respectively) T-DNA have been detected, which implies that a simple excision of plasmid T-DNA is not observed. A specific direct repeat sequence of 25 base pairs defines the ends of the T-DNA and determines the direction of the transfer.

The main drawback of the *Agrobacterium*-mediated transfer is that not all plants are susceptible to the pathogen. A wounding response is necessary, and only dicotyledonous show a pronounced response. Monocotyledonous (which include all cereals) in general are not susceptible to *Agrobacterium*. Differentiated cereal tissue does not dedifferentiate in response to infection; in general, cells die instead of dedifferentiating, although the time elapsed between infection and death permits, in some cases, transfer of T-DNA to cells (Grimsley et al., 1987).

Comprehensive surveys of *Agrobacterium* infectivity of over a thousand angiosperm and gymnosperm plants (De Cleene and De Ley, 1976; De Cleene, 1985) revealed that only 3% of the monocotyledoneous plants were host to the bacterium, whereas 60% of dicotyledoneous and gymnosperms were susceptible to infection.

Asparagus officinalis L. was the first monocotyledonous plant from which hormone-independent and opine-producing crown gall tissue was isolated (Bytebier et al., 1987). More recent studies showed stable transformation of important cereals by *Agrobacterium*, including rice (Chan et al., 1992; Rainieri et al., 1990), wheat (Mooney et al., 1991) and maize (Gould et al., 1991; Rainieri et al., 1993).

Research on the causes of the lower levels of transformation of monocotyledoneous plants, compared to dicotyledoneous plants, revealed the importance of plant

metabolites in the induction of the *vir* genes. For example, Sahi et al. (1990) demonstrated that a secondary maize metabolite inhibits the induction of the *vir* genes, which could account for the lower rates of transformation in maize. It has also been demonstrated that plants known to be typical phenol accumulators are more susceptible to infection (De Cleene and De Ley, 1981).

Rainieri et al. (1993) showed that the VirA protein of different *Agrobacterium* strains differ; some of the allelic forms of the *vir*A gene are not sensitive to the substances released by monocotyledoneous plants. Octapine- and nopaline-type Ti plasmids also differ in the efficiency of transformation of grasses (Chilton, 1993). VirA produced by nopaline-type Ti plasmids appears to work well in maize, but that of octapine-type plasmids is inefficient, which shows the importance of selection of the right bacterial strain. In addition, a mutant *vir*G gene causing constitutive expression of the *vir* operon increased the transformation efficiency (Hansen et al., 1994). Gene transfer to maize by the octapine strain LBA4404 improved upon introduction of that mutation, whereas multiple copies of the wild-type *vir*G were ineffective, but worked well in tobacco.

Furthermore, molecular characterization of the *virF* locus of *A. tumefaciens* (Melchers et al., 1990) also revealed that octapine and nopaline strains differ in virulence in *Nicotiana glauca* Grah. The nopaline Ti plasmids

lack a functional *virF* gene, which codes for a hydrophilic protein. The authors suggest that, if one considers using the *Agrobacterium*-delivery system for plant transformation, one should first find out which virulence system is optimally adapted to the plant species involved.

Usually, one gene of interest is used in each tranformation event. Integration of multiple transgenes can also be accomplished, either by introduction in tandem, or by co-infection of the same cell by bacteria carrying different transgenes (McKnight et al., 1987). An alternative approach was investigated by Brasileiro et al. (1991): transformation was promoted by co-inoculation with a tumor-inducing strain and a disarmed strain providing the gene or genes of interest. The method takes advantage of the natural shoot regeneration capacity in tumors induced by wild-type *Agrobacterium*, and showed a non-requirement for a regeneration procedure, a short culture period, few *in vitro* manipulations, and high efficiency in poplar, a tree species.

Silicon Carbide Fiber-Mediated Transformation

In this system, silicon carbide fibers, plant cells, and plasmid DNA containing the sequences of interest are mixed together, then accelerated, usually by vortexing the mixture. The fibers are able to puncture the cells, facilitating DNA uptake. In principle, the system is very

simple and does not require special equipment. Kaeppler et al. (1990) pioneered this system for delivery of DNA into cells of tobacco and maize. In this experiment, the authors observed that fibers apparently penetrated the cell walls, suggesting that DNA adhered to the fibers and was discharged through the cell wall into the cytoplasm and nucleus. Successful transient and stable transformation of several species using the silicon carbide fibers-DNA delivery system has been reported (e.g., Dunahay, 1993; Kaeppler et al. 1990, 1992).

Virus Resistance in Plants

In plants, cross protection is a method used to confer resistance to viruses. This is achieved by inoculating the crops with a mild strain of the virus. Some of the drawbacks of cross protection include the necessity for large-scale field infections with the mild strain, and the reduction in yield which is generally associated with the infection (Gonsalves and Fulton, 1977).

In contrast, the hypersensitive response triggered by an incompatible plant-pathogen interaction tends to be nonspecific (Fraser, 1990). One general effect of this response is the production of ethylene, a plant hormone able to induce many stress responses. In addition to inducing a defense aimed at impounding the pathogen, various antimicrobial compounds are synthesized. These induced

responses contribute to an active defense mechanism of the plant known as induced resistance, which occurs as well in response to virus infections.

One important component of resistance is the pathogenesis-related (PR) proteins. Induction of PR proteins by abiotic agents, such as salicylic acid, some amino-acid derivatives, and barium and manganese salts, also can result in acquired resistance to pathogens. The host proteins induced by the hypersensitive response of plants to infection seem to be involved in (i) a direct attack of the invading pathogen; (ii) a limitation of the pathogen at the site of infection through lignification; and (iii) an adaptation of the host metabolism to the stress condition (for review of these various aspects see Bol et al., 1990).

Genetically-engineered virus resistance via the use of cloned viral genes is a direct result of the progress in molecular genetic and plant transformation techniques in the last few years. The advantages of using viral genes, as opposed to host genes, is that potentially useful viral genes can be identified, isolated, and cloned more easily (Grumet, 1990).

This approach also takes advantage of the principle that in any host-pathogen interaction, there are certain pathogen-encoded functions that are essential to the pathogen, but not to the host. If one of these functions is disrupted, the pathogenic process should stop. Two potential

advantages of this are as follows: (i) the source of resistance genes would not be questioned, since each pathogen would provide resistance genes; and (ii) pathogen genomes are generally small, and the isolation of the genes should be simpler (Grumet, 1990). Genetically-engineered virus resistance has been demonstrated in several plant and animal systems; some of them will be discussed in more detail later.

A Variety of Genetic Materials Can Be Used to Transfer Virus Resistance Genes

Coat protein-mediated resistance is the most common method in genetic engineering for virus resistance. Only genes responsible for the coat protein (CP) are included in the construct. Other approaches include the use of satellite sequences, antisense RNAs, and the whole genome of a mild virus. Yet other strategies, such as interference with viral replication by sense RNAs or by defective interfering particles, or the use of transgenic plants that constitutively express pathogenesis-related proteins, have been suggested (Grumet, 1990). I shall deal briefly with some of the most promising alternatives, and then concentrate on virus CP-mediated resistance.

Complete viral genomes from mild strains. In some examples with tobacco mosaic virus (TMV), the use of a complete viral genome from a mild strain resulted in better

protection than the use of CP genes; inoculation with naked RNA or with a high titer of the inoculum did not overcome the complete genome-conferred protection, but did overcome the resistance conferred by CP. In other examples (Grumet, 1990), transgenic plants expressing either virus CP genes only or whole viral genomes had the same level of resistance.

Some prerequisites for the use of whole genome from mild stains are as follows: (i) a stable mild strain must be available; (ii) the introduction of the genome in the plant should not cause any decrease in productivity or in quality; and (iii) the mild strain must not mutate to a virulent strain (Yamaya et al., 1988). The size of the genome may also impose some restrictions. In a way, these are the same drawbacks encountered in the use of cross-protection by infecting plants in the field with a mild strain of the virus.

Antisense sequences. Antisense RNAs that complement the messenger RNA strand have been used to inhibit gene expression, probably due to hybridization of the two complementary strands limiting the access of ribosomes; the RNA-RNA hybrids may also be selectively degraded. It is possible that the antisense sequences also interfere with replication of the viral genome. However, the success of this technique has been limited (Grumet, 1990).

Satellite sequences. Viewed as virus parasites, satellite sequences are small, single-stranded RNA molecules that are dependent on the host virus for replication; they can only increase in a host plant that is also infected by a a closely related virus. There are two groups of satellite sequences: satellite RNAs, which are unable to encode their coat protein and accumulate in capsids composed of the coat protein of their supporting virus; and satellite viruses, which encode their own coat protein (Bruening et al., 1991). They do not share nucleotide sequence with the host and are not required for virus replication.

Only a limited number of RNA viruses contain satellite sequences, and, in some cases, the presence of the sequences diminishes the symptoms of the disease. One of the possible explanations is that the supporting virus titer may be reduced in the presence of the satellite due to sequestration of coat protein (Bruening et al., 1991). Unfortunately, the effects of the presence of satellite sequences are not predictable; there are instances in which the symptoms are made worse due to the presence of satellites (Kurath and Palukaitis, 1989; Waterworth et al., 1979). Therefore, features that may preclude the widespread use of satellite RNA in agriculture include the variable nature of the satellite RNAs, and the fact that they have not been identified for the majority of plant viruses (Beachy, 1991).

Virus coat protein. The use of the gene that codes for the virus coat protein seems to be the most promising approach. The coat protein gene is combined with a reporter gene and with a gene coding for resistance to some antibiotic. The construct is then transferred to explants of the desired species, usually by means of an *Agrobacterium*-mediated system.

There Are Several Steps to Create Coat Protein-Mediated Resistance

In order to isolate the required gene or genes, an extensive knowledge of the genomic organization of the virus is required. The epidemiology and symptomatology of the virus disease are also important, as is the availability of a suitable system to isolate and transfer the desired portion of the genome to the plant (Beachy et al., 1990). As already discussed, *Agrobacterium*-mediated systems are suitable for many plants, and have been used extensively for the creation of CP^+ plants.

Construction of chimeric genes. The first step is the isolation of the viral genetic material, usually RNA, which is used as a template for reverse transcription to produce a complementary DNA (cDNA) copy of all or part of the genome. For many viruses, CP is encoded by subgenomic RNA, and the isolation of cloned cDNA that represents the open reading frame (ORF) for the CP is an easy process. However, for

those viruses where the CP is part of a larger polyprotein, the process is more difficult. Other steps may be necessary after isolation of the cDNA clone, such as the elimination of sequences that potentially may affect stability or interfere with the translational process. Efficient translation has to be guaranteed, and in some cases, modifications of the base sequence have to be made.

An appropriate transcriptional promoter has to be included, since plant RNA viruses do not contain promoters capable of transcription in plant chromosomes. It is known that maximal expression of foreign genes may require gene constructs that are fashioned with promoters and terminators that allow maximum expression (Narvaez-Vasques, 1992). One such promoter, 35S, derived from cauliflower mosaic virus, is extensively used when a strong promoter is desired. Another important step is including a 3'-end sequence for termination and polyadenylation of the transcripts. This allows the translation of the DNA sequence we are interested in. Many sequences are available, derived from various sources.

Usually, a reporter gene is included in the construct, to facilitate the detection of transformed cells or plants. An antibiotic-resistance gene is frequently included, to allow preferential growth of transformed cells.

Introduction of the chimeric gene into plants. Upon isolation of the gene, it is transferred to a vector for

introduction into the plant genome. *A. tumefaciens* is frequently used. Engineered, disarmed strains have been produced that allow the bacteria to introduce the CP gene and any reporter or antibiotic-resistance genes, instead of its normal pathogenic genes.

Expression of resistance. The final objective of the process is to obtain transformed plants (CP^+) that will have some advantage over non-transformed plants upon virus infection. Usually, not only the directly-transformed plants are tested, but also crosses are made to assess the inheritance of the resistance. Segregation can be assessed directly, by infecting the plants (Quemada et al., 1992), but also immunological techniques can be used, based on antibodies to coat proteins; a reporter gene that has been introduced with the CP gene can likewise be followed.

The resistance is usually expressed in one of the following categories: (i) fewer infection sites are present in infected organs; (ii) less systemic disease develops upon infection; (iii) fewer viral particles accumulate in CP^+ plants. This can be assessed by ELISA, by immunoblots or by direct visualization of virus particles under electron microscope. In some cases, the amount of protection is correlated with the amount of CP produced, and it may be beneficial to introduce more than one copy of the gene, as well as to use stronger promoters. The levels of viral coat protein generally range from 0.01 to 0.3% total soluble

protein in the cells, depending on the virus, the promoter, the number of genes inserted and the site of insertion in the host genome (see for instance Jongedijk et al., 1992).

The Mechanisms of CP-Mediated Resistance Are Complex and not Fully Understood

Although very successful results in the use of virus coat protein in diminishing the negative effects of viruses have already been reported, the mechanisms underlying the resistance are not always straightforward. The mechanism of the infection is a complex event, and a good understanding of it can lead to a better understanding of the mechanisms for resistance.

Early events in infection. The first event after virus infection is the release of the viral nucleic acid for translation. In some cases a swelling of the virion is observed. This is followed by binding of ribosomes and disassembly of the virus concurrent with translation (Beachy et al., 1990). It appears that the expression of the CP genes interferes with an early event in infection. This can be demonstrated by some examples in which inoculation of plants with uncoated viral nucleic acid overcome resistance in CP^+ plants which showed resistance when infected with whole virions.

These experiments support the theory that CP interferes with an early event in infection that releases the

encapsidated nucleic acid. However, which event is primarily affected is not yet known. Reencapsidation of viral genetic material as a primary mechanism for all plants and virus can be ruled out.

Other mechanisms that act after the uncoating of the viral particles have been implicated. Examples are found in which a consistent, although low, level of resistance to infection by viral nucleic acid is observed (Osbourn et al., 1989). The most logical mechanism would be interference with the replication of the viral genetic material.

Protection against infection is effective not only for the virus that served as donor of the CP gene but also to related viruses showing some similarity. It was shown (Nejidat and Beachy, 1990) that protection was conferred to viruses showing as little as 60% of base sequence similarity.

The accumulation of coat protein was believed to be a requirement for coat protein-mediated protection (Lal and Lal, 1993). In some experiments, chimeric genes that encoded sequences representing the CP subgenomic RNA but did not produce protein due to removal of the initiation codon, were shown to be ineffective (Powel et al., 1989). This illustrated that CP, and not RNA, was responsible for the resistance to infection. However, more recent data suggest that the mechanisms are more complex and certainly more

diverse, with the involvement of RNA in many cases [see review by Hackland et al. (1994)].

Later events: spread of infection. It has been shown that the systemic spread of virus infection was prevented or delayed in CP^+ plants, which could result from interference with (i) the cell-to-cell spread in inoculated tissue, (ii) the egress of virus particles from the inoculated organ into the vascular tissue, (iii) the movement of the particle once inside the vascular system, (iv) the ingress into non-inoculated leaves, (v) the initiation of infection in other leaves, or a combination of two or more of these (Beachy et al., 1990). In one example, spread to distant tissue was significantly reduced in CP^+ plants, although the spread of virus to closely adjacent tissue (1-3 mm) was similar in CP^+ and control plants (Wisniewski et al., 1990).

Peanut Stunt Virus (PSV)

Peanut stunt virus, also known as groundnut stunt virus, is a single-stranded (ss), positive-sense RNA virus with isometric particles about 30 nm in diameter. It is a member of the cucumovirus group, all of which have a capsid protein composed of a single polypeptide of approximately 24kDa of mass. PSV genome is tripartite, composed of ss-RNA 1, 2 and 3 species; a fourth species (RNA 4) codes for the virus coat protein, and is also encapsidated with the other

species. The nucleotide sequence for a cDNA encoding for the PSV-CP is given by Naidu et al. (1991a).

Some isolates of PSV contain a satellite RNA (satRNA), called PSV-associated RNA 5 (PARNA 5). This is composed of ss-RNA, 393 nucleotides long, with no sequence homology with the viral genome. The molecular weight of the satellite is 1.27×10^5. It is capped with M7Gppp at the 5'-end, and has an hydroxyl group at the 3'-end. It is encapsidated with the viral RNA and replicates only in the presence of the viral RNA (Collmer et al., 1985; Dias-Ruiz et al., 1987; Linthorst and Kaper, 1984). A ds form of this satRNA accumulates in large amounts during infection, and probably represents a replicative form (RF). Most of this ds fraction, as well as the ds viral RNA present in infected tissues, is associated with a uniform vesicular fraction, found also in healthy plant tissues. The vesicles are probably the site for in vivo satellite and viral replication (Dias-Ruiz et al., 1987).

Three variants of PSV satRNA, (V)-, (G)- and (WC)-satRNA, have been isolated (Naidu et al., 1991b). They differ in the way they interact with viral RNA in the development of disease symptoms; (V)-satRNA does not affect symptomatology, whereas (G)- and (WC)-satRNA attenuate the symptoms in tobacco. Molecular characterization of the nucleotide sequences of these variants revealed that the (G)-satRNA differs in two positions with the published

sequence (Collmer et al., 1985), one being a nucleotide transition of G to T at position 166, and the other being a nucleotide transversion of T to C at position 247. (G)- and (WC)-satRNA showed six- and eight-position differences, including one and two deletions, respectively.

Analysis of progeny viral RNA from plants challenged with PSV with or without the satRNAs showed that less viral RNA accumulated in the presence of (G)- or (WC)-satRNA, compared to satellite-free PSV-inoculated plants. This seems to support the competition mechanism proposed by Piazzola et al. (1982), i.e., that the satRNA competes with viral RNA for binding to the RNA replicase, resulting in reduced viral replication and attenuated symptoms.

PSV has been reported in the United States (Ahmad and Scott, 1985; Anderson et al., 1991; McLaughlin, 1986; McLaughlin and Boykin, 1988), China (Zhang et al., 1990), Poland (Frencel and Pospieszny, 1985), Indonesia (Green et al., 1988), Korea (Kim et al., 1988), Spain (Diaz-Ruiz et al., 1979), and Sudan (Ahmed, 1986; Ahmed and El Sadiq, 1985; Ahmed and Mills, 1985), among other countries.

Variant strains differing in one or more host reactions have been reported. Two strains were initially described. The Eastern strain (PSV-E), found in Southeastern USA, produces necrotic primary lesions, systemic necrotic rings and veinal necrosis in bean, and mosaic without necrosis in pea. In gel-diffusion tests, this strain produces a single

curved band near the antigen well (Gooding, 1968; Kuhn, 1969). The Western strain (PSV-W), initially found in the state of Washington, produces mosaic without necrosis in bean, and mosaic with severe necrosis of stems and petioles in pea. This strain produces a curved band near the antigen well and one or more straight bands near the antiserum well (Mink et al., 1969). Subsequently, other variant strains have been described. Strains PSV-H and PSV-T are closely related serologically to the Eastern strain, strains PSV-J and PSV-B are closely related to the Western strain, and strain PSV-V is closely related to, but serologically distinct from PSV-E. PSV-Tp, isolated from *Trifolium pratense* L., is distinct from both PSV-E and PSV-W [see Xu et al. (1986) for a review].

Physical, serological and chemical properties of PSV are indicative of some affinity with cucumber mosaic virus and tomato aspermy virus. Nonetheless, since strains of all three viruses differ among themselves, the degree of relationship is dependent upon the strains being compared (Mink, 1972).

Symptomatology, Transmission and Control

PSV causes pronounced stunting of peanuts (*Arachis hypogea* L.), as well as malformation of the fruits. Besides peanuts, the virus also infects many other dicotyledonous species in the Leguminosae, Chenopodiaceae, Compositae,

Cucurbitaceae and Solanaceae families, such as beans (Mink et al., 1969), tobacco (Gooding, 1968), clovers (Campbell and Moyer, 1984; Ragland et al., 1986), *Dolichos lab-lab* L. and *Clitorea sp.* (Ahmed and Mills, 1985), alfalfa (Ahmed and Mills, 1985; Ahmed et al., 1985), *Vicia faba* L. (Ahmed, 1986) and soybeans (Buss et al., 1987, 1988).

PSV is transmitted by aphids in a non-persistent manner and is readily transmissible by inoculation of sap. It has also been found to be transmitted by dodder. *Aphis craccivora*, *A. spiraecola*, *A. solanella*, *Lipapis erysimi* and *Myzus persicae* are the most common vectors; it is not transmitted by *Aphis gossypii* nor by *Aulacorthum solani* or *Rhopalosiphum maidis* (Ahmed and El Sadiq, 1985).

Viruliferous aphids retained the virus for 30 minutes, but pos-acquisition starvation for more than 30 minutes resulted in almost complete loss of the virus. Control of the vectors has proven to be an effective measure to reduce the extent of the infection (Smith and Culp, 1985).

The virus is not transmitted to aphid progeny, and is hardly (less than 0.1%) transmitted through infected peanut seeds (Kuhn, 1969). In the event of seed transmission, the seedlings are late-emerging and present poor growth, so seed transmission is not considered an important factor in disease spread in peanut fields. However, it was found to be transmitted by seeds of yellow and blue lupine.

Cowpea [*Vigna sinensis* (L.) Savi], French bean (*Phaseolus vulgaris* L.), jimson weed (*Datura stramonium* L.), tomato (*Lycopersicum esculentum* L.), tobacco (*Nicotiana tabacum* L.), *Chenopodium amaranticolor* Coste et Reyn, and *C. quinoa* Willd are the most common diagnostic and assay species.

When cowpea is used for maintaining PSV and as a source of virus for purification, leaves 7-8 days after inoculation can be used. The purification steps include Steere's chloroform-butanol method, differential ultracentrifugation and rate zonal sucrose density-gradient centrifugation. Conditions during purification determine both yield and activity of the sap (Mink 1972). Once the sap has been obtained, its longevity is strongly influenced by storage conditions. At room temperature, infectivity can be lost after 4-24 h, depending upon the plant used to obtain the sap. At higher temperatures (50 - 60 °C), infectivity can be lost in as little as 10 min. Addition of antioxidants or reducing agents helps in preserving the infectivity for several days.

Kanamycin and Geneticin as Selectable Antibiotics in Plant Transformation Experiments

Kanamycin and geneticin are aminocyclitol-containing aminoglycoside antibiotics. This group of antibiotics is characterized by two or more amino sugars joined in glycosidic linkage to a hexose nucleus, which is usually in

a central position. This hexose, or aminocyclitol, is either streptidine, as in streptomycin, or 2-deoxystreptamine, as in all other antibiotics in this group (Sande and Mandel, 1990). Among the disaccharide-containing aminoglycosides, neamine (or neomycin A) and paromamine can be cited. The oligosaccharide-containing aminoglycosides are subdivided in the following groups: Streptomycin (e.g. streptomycin, glebomycin); Kanamycin (e.g. kanamycins A, B and C, tobramycin); Gentamicin (e.g. gentamicins C1, C2, and A, sisomicin); Neomycin (e.g. neomycins B and C, paromomycins A and B, ribostamycin); Destomycin (e.g. destomycins A and B, hygromycin B); and the Spectinomycin group (spectinomycin or actinospectacin). Other aminoglycoside antibiotics include kasugamycin and validamycin, both with a cyclitol group instead of an aminocyclitol, and nojirimycin and streptozocin, which contain neither aminocycolitol or cyclitol groups (Tanaka, 1975).

Kanamycin is isolated from *Streptomyces kanamyceticus*, which produces also two other minor components, kanamycins B and C; for this reason, kanamycin is also referred to as kanamycin A. The antimicrobial activity of these three compounds differ (Tanaka, 1975). The first description of this antibiotic was made by Umezawa et al. (1957), cited by Weinstein and Wagman (1978). Kanamycin is a bacteriocide that acts by inhibiting protein synthesis and translocation, and eliciting miscoding (Ausubel et al., 1991).

Geneticin, also known as antibiotic G418 sulfate, is not normally used as a standard antibiotic; its most common application is as a selection agent in molecular biology. Geneticin is a 2-deoxystreptamine antibiotic, and is toxic to bacteria, yeast, protozoa, helminths, and mammalian cells (Sigma, 1994). The structure of G418 resembles that of gentamicin, neomycin and kanamycin but, unlike these related compounds, it interferes with the function of the 80S ribosomes and blocks protein synthesis in eukaryotic cells (Davies and Jimenez, 1980; Southern and Berg, 1982). The concentration of G418 required for inhibition of these various organisms varies from 5 $\mu g\ mL^{-1}$ or less for bacteria and algae to 300-500 $\mu g\ mL^{-1}$ for animal cells; on the other hand, resistant colonies of *Saccharomyces cerevisiae* transformed with *Tn*601 constructs grew in the presence of more than 1,000 $\mu g\ mL^{-1}$ of G418 (Davies and Jimenez, 1980).

The aminoglycoside antibiotics affect peptide chain initiation by blocking the 30S initiation complex formation, by inducing breakdown of the 70S initiation complex, and/or by inhibiting ribosomal dissociation. Kanamycin, neomycin, paromomycin and streptomycin have also been shown to disturb the fidelity of translation of the genetic code *in vivo*, and a similar effect has been observed *in vitro* with these antibiotics as well as hygromycin B and gentamicin. Besides acting on prokaryotes, several aminoglycosides also have

marked effects on eukaryotic cells (Davies et al., 1965; Gale et al., 1981; Gorini and Kataja, 1964).

The resistance to aminoglycoside antibiotics in bacteria can be classified into several groups of biochemical mechanisms: (i) alteration of the target site in the cell that reduces or eliminates the binding of the drug to the target site; (ii) blocking the transport of the antibiotic into the cell; (iii) detoxification or inactivation of the antibiotic; (iv) providing the cell with a replacement for the metabolic step that is inhibited, a by-pass mechanism; (v) increasing the concentration of the enzyme inhibited, a titration-out mechanism; (vi) production of a metabolite that can antagonize the inhibitory effect of the inhibitor; and (vii) decreasing the cell's metabolic requirement for the pathway or reaction inhibited by the drug (Davies and Smith, 1978).

The most common mode of resistance to amynoglycoside antibiotics depends on the presence of plasmid-coded modifying enzymes, which are classified according to the modification they induce on the molecules: N-acetylation (e.g. AAC, N-acetyltransferases), O-phosphorylation (e.g. AAD, O-nucleotydiltransferases), or O-nucleotidylation (e.g. APH, O-phosphotransferases; Davies and Smith, 1978). Several enzymes have been characterized which are able to inactivate kanamycins, for example kanamycin phosphotransferase I and II (APH class), kanamycin adenyltransferase (AAD class), and

kanamycin acetyltransferase (AAC class). Besides acting on kanamycins, these enzymes also inactivate several other aminoglycoside antibiotics, including geneticin. Resistance to neamine (*neaA* , *rpsE* and *rpsL* strains) results from alterations to ribosomal proteins S5, S12 and S17 (see review by Tanaka, 1975). Other enzymes implicated in resistance to this class of antibiotics include hygromycin phosphotransferase and puromycin acetyltransferase, coded by the genes *hyg* and *pac*, respectively (Luna and Ortiz, 1992).

Resistance to geneticin is conferred by one of two dominant genes of bacterial origin which can be expressed in eukaryotic cells (Colbere-Garapin et al., 1981). Jimenez and Davies (1980) have shown that yeasts are sensitive to G418, but resistance can be conferred in clones transfected with the *Tn*601 transposon; this transposon codes for aminoglycoside 3'-phosphotransferase type I (APH-3'-I), that phosphorylates and inactivates this and other aminoglycoside antibiotics containing the 2-deoxystreptamine moiety, at the 3'-hydroxyl position (Jimenez and Davies, 1980). Davies and Smith (1978), and Chen and Fukuhara (1988) also report *Tn*6 and *Tn*903, respectively, as sources for this enzyme.

Resistance to geneticin is also conferred by aminoglycoside 3'-phosphotransferase type II (APH-3'-II), carried by transposon *Tn*5 (Jorgensen et al., 1979), not only in bacteria but also in human, simian and murine cell lines (Colbere-Garapin et al., 1981). The phosphotransferase gene

from *Tn*5 is designated *neo* (Southern and Berg, 1982). This gene provides, in fact, resistance to the kanamycin and neomycin groups of antibiotics (Berg et al., 1978). *Tn*5 is a 5,400 base-pair-long transposon, but it has been shown that a 1,500 bp region is sufficient for conferral of resistance (Jorgensen et al., 1979). Other genes that confer resistance to geneticin include those responsible for the production of the enzymes APH-2" (2"-phosphotransferase), AAC-2' (2'-acetyltransferase), AAC-3-I and AAC-3-IV (3-acetyltransferase), and ANT-2" and ANT-4' (2"- and 4'-adenyltransferase, respectively)(Davies and Jimenez, 1980).

The use of kanamycin, geneticin and other aminoglycoside antibiotics in plant systems

Chimeric genes for the expression of the *Tn*5 neomycin phosphotransferase II gene (*npt*II) have become the standard selectable marker for plant transformation vectors. The kanamycin resistance provides for the direct selection of transformants in a wide variety of plants (Rogers et al., 1987). In red clover, it has been shown that callus growth was affected at concentrations as low as 5 $\mu g\ mL^{-1}$, and showed total inhibition of growth at concentrations above 25 $\mu g\ mL^{-1}$ kanamycin added to the tissue culture medium; concentrations of 50 $\mu g\ mL^{-1}$ of this antibiotic are routinely used for selection of red clover transformants carrying the *npt*II gene (Quesenberry et al., 1992).

Kanamycin has been extensively used as a selection

agent in plant transformation experiments in which the *npt*II gene was used. Geneticin traditionally has been used in animal cell cultures, but more recently its use has been extended to plant systems, replacing or complementing kanamycin. The effects of these and other aminoglycoside antibiotics vary, depending upon several factors, including concentration and plant genotype. For a given protocol, kanamycin is usually less toxic than geneticin to plant cells. For instance, Catlin (1990) found that sugarbeet callus growth was inhibited by levels of 50 $\mu g\ mL^{-1}$ geneticin, as opposed to 150 $\mu g\ mL^{-1}$ kanamycin; other aminoglycoside antibiotics tested by this author were gentamicin, hygromycin, and phleomycin, with the following inhibitory doses, respectively: 150 $\mu g\ mL^{-1}$, 10 $\mu g\ mL^{-1}$, and 20 $\mu g\ mL^{-1}$. In the same experiment, total inhibition of shoot formation from callus was achieved at kanamycin concentrations as high as 150 $\mu g\ mL^{-1}$, whereas 10 $\mu g\ mL^{-1}$ geneticin were able to completely inhibit shoot formation. Apple propagation from leaf tissue was inhibited by 1 $\mu g\ mL^{-1}$ geneticin, 5 $\mu g\ mL^{-1}$ kanamycin, 10-25 $\mu g\ mL^{-1}$ paromomycin, or 100 $\mu g\ mL^{-1}$ neomycin, whereas *npt*II-transgenic plants had an increased resistance to all four antibiotics, with inhibition of regeneration occurring at 2.5 $\mu g\ mL^{-1}$ geneticin, 100 $\mu g\ mL^{-1}$ kanamycin, 375 $\mu g\ mL^{-1}$ paromomycin, and 375 $\mu g\ mL^{-1}$ neomycin (Norelli and Aldwinckle, 1993).

Rice has also demonstrated much greater sensitivity to geneticin than to kanamycin (Dekeyser et al., 1989).

However, Orlikowska et al. (1995) found that kanamycin was a more potent inhibitor of safflower callus development than geneticin; concentrations of 75 μg mL^{-1} geneticin and 60 μg mL^{-1} kanamycin had the same inhibitory effect. In *Arabidopsis thaliana*, 20 μg mL^{-1} geneticin and 25 μg mL^{-1} kanamycin are used for selection of transgenic plants (Schmidt and Willmitzer, 1988). Firoozabady et al. (1994) observed that chlorsulfuron was superior to both kanamycin and geneticin as a selectable antibiotic in carnation transformation experiments.

Tsang et al. (1989) tested the toxicity of various aminoglycoside antibiotics on zygotic embryos of *Picea glauca* (Moench) Voss, a woody species, and found that methotrexate inhibited 85% and 100% of embryo formation at concentrations of 2.5 and 5.0 μg mL^{-1}, respectively. Geneticin and hygromycin reduced 75% and 80% bud formation at 5 μg mL^{-1}, respectively, and 100% at 10 μg mL^{-1}, whereas kanamycin reduced bud formation by 90% at 10 μg mL^{-1}, and 100% at concentrations of 20-50 μg mL^{-1}. In this protocol, concentrations of 100-200 μg mL^{-1} cefotaxime exhibited no toxic effects. In addition, the authors observed that 2-day-old cultures were more sensitive than 9-day-old cultures to the antibiotics.

Kanamycin and geneticin have also been used in combination, to increase the efficiency of selection. Seki et al. (1991) used a protocol for regeneration of transformed *Arabidopsis thaliana* Heynh. plants in which the initial phases of tissue culture are carried out in medium containing 50 $\mu g\ mL^{-1}$ kanamycin; this concentration was decreased to 20 $\mu g\ mL^{-1}$ after 10 days. Calli were then transferred to medium containing 10 $\mu g\ mL^{-1}$ geneticin, and final selection of transformants is carried out in medium containing 20 $\mu g\ mL^{-1}$ geneticin.

In general, paromomycin has been superior to other aminoglycoside antibiotics for selection of *npt*II-transgenics. It has been successfully used in citrus (Vardi et al., 1990), rapeseed (Guerche et al., 1987), sunflower (Escandon and Hahne, 1991), oat (Torbert et al., 1991), and tobacco (Bellini et al., 1989). Norelli and Aldwinckle (1993) found that paromomycin elicited a much grater differential between the sensitivity of transformed and non-transformed plants, however, concentrations of paromomycin necessary for the selection of transformed plants resulted in the necrosis of nontransgenic plants, which may be inhibitory to regeneration. In this experiment, this antibiotic also presented growth-regulator properties when used at doses below the inhibitory concentration.

Norelli and Alswinckle (1993) found variable responses of apple explants to kanamycin. In one experiment,

regeneration was inhibited at 10 μg mL^{-1}, but in another experiment, regeneration was not inhibited until a concentration of 100 μg mL^{-1} was used.

It has also been shown that antibiotics may have growth regulator-like effects. Norelli and Aldwinckle (1993) tested the effects of several concentrations of aminoglycoside antibiotics in the regeneration and selection of transgenic apple tissue expressing the *npt*II gene, and observed that paromomycin and neomycin increased the percent regeneration at doses below the inhibitory concentration, whereas kanamycin and geneticin did not. Cefotaxime, a ß-lactam antibiotic that inhibits cell wall synthesis in bacteria, and is used to eliminate *Agrobacterium* in transformation experiments, has also been found to promote apple regeneration (James et al., 1990). Catlin (1990) observed similar effects for kanamycin and phleomycin. Levels of 10, 20 and 30 μg mL^{-1} kanamycin increased 109, 108 and 75% the number of shoots recovered from sugarbeet tissue culture, whereas it had almost no effect on the number of explants forming callus. Geneticin tested in the same experiment did not have a positive effect on growth, and was able to totally inhibit shoot formation at concentrations of 10 μg mL^{-1}.

Yepes and Aldwinckle (1994a) observed that cefotaxime at 250 μg mL^{-1} enhanced regeneration and shoot development in apple explants, and carbenicillin at 500 μg mL^{-1} induced

abundant callus formation but inhibited regeneration. In another experiment (Yepes and Aldwinckle, 1994b), cefotaxime at 200 µg mL^{-1} stimulated shoot growth and development, but at 500 µg mL^{-1} caused abnormal shoot morphology. Carbenicillin at 500 µg mL^{-1}, alone or with 200 µg mL^{-1} cefotaxime, inhibited proliferation, caused callus formation, and release of phenolics into medium. Norelli and Aldwinckle (1993) found a broad spectrum of tolerance of several apple genotypes to aminoglycoside antibiotics, some genotypes being 38 times more resistant than others to paromomycin, 2.5 times to geneticin, and 3.8 times to neomycin.

Acetosyringone and the *vir* Genes of *A. tumefaciens*

Stachel et al. (1985) showed that the expression of the *vir* operon is activated by the phenolic plant molecules AS and alpha-hydroxyacetosyringone (OH-AS), which are produced in exudates of wounded and metabolically-active plant cells, allowing *Agrobacterium* to recognize susceptible cells in nature. A cascade of events, starting from the activation of *vir*A and *vir*G, leads to the transfer of T-DNA into the plant nucleus, with subsequent integration into the plant genome [for a review of the events, see Tinland and Hohn (1985)].

Relative virulence of *Agrobacterium* strains may be important in determining the efficiency of plant transformation. In apple, Dandekar et al. (1990) identified

several factors that influence virulence, including (i) the strain of *Agrobacterium*, (ii) the spatial location of wounds on leaf tissue, (iii) the leaf position on the shoot, (iv) replication regions on introduced binary plasmids, and (v) number of copies of the transcription activator *vir*G gene on a plasmid. *Agrobacterium* strains were also a key factor in determining the efficiency of transformation of *Pisum sativum* L. (Puonti-Kaerlas et al., 1989). The response patterns of five cultivars of this horticultural species coincided largely, being more dependent on the bacterial strain than on the cultivar used. On the other hand, Lewis and Bliss (1994) found a wide range of genotypic differences in susceptibility to transformation by *Agrobacterium* when several cultivars of commom bean were tested.

Besides the factors mentioned, chemical and environmental factors in the virulence-inducing medium in which *Agrobacterium* is grown prior to or during transformation can also influence the efficiency of plant transformation. Phenolics, sugars, temperature and pH are included among those factors. Jin et al. (1993) showed that virulence gene expression is specifically inhibited at temperatures above 32 °C, confirming the findings that *Agrobacterium* is only able to cause tumors at lower temperatures.

Acetosyringone (AS; 3'5'-dimethoxy-4'hydroxyacetophane) has been implicated as an important phenolic compound for

the induction of virulence in *Agrobacterium*. The ability to respond to this compound is provided by the Ti-plasmid-encoded system comprising VirA and VirG, products of *vir*A and *vir*G, the *vir* regulatory genes (Palmer and Shaw, 1992). VirA is a membrane-bound sensor kinase protein, and VirG is a cytoplasmic regulator protein. Pan et al. (1993) provided physical evidence that VirA exists as a homodimer in its native configuration, and that the dimerization neither requires nor is stimulated by AS.

Other compounds, including synthetic AS, acetovanillone, syringaldehyde and syringic acid ß-glycosides have been tested for their ability to induce the *vir* operon using a *vir*E::*lac*Z fusion plasmid (Delmotte et al., 1991). Acetosyringonyl ß-L-fucopyranoside was the most active monoglycoside tested, however monoglycosides were less active inducers than free AS. In contrast, the beta-maltoside of syringaldehyde showed higher activity than the free phenol at higher concentrations, which could be related to specific sugar receptors on the bacterial cell surface.

In addition to the well-established effects of VirA and VirG on activation of the *vir* operon, more recent studies showed that other proteins that are not tumor-inducing plasmid encoded probably mediate *vir* gene activation in a step prior to the VirA/VirG two-component regulatory system (Lee et al., 1992). The product of the chromosomal gene

*mia*A, probably a tRNA isopentenyltransferase, has also been implicated (Gray et al., 1992)

Primich-Zachwieja and Minocha (1991) tested extracts from 44 plant species belonging to different taxa for their ability to induce the expression of the virulence *vir*E gene in *Agrobacterium* containing a *vir*E::*lac*Z fusion construct, and found that most of the plant extracts, including those from algae, bryophytes, pteridophytes, gymnosperm, monocotyledonous and dicotyledonous, induced the expression of the *vir*E gene, as detected by the presence of beta-galactosidase activity in the bacteria.

Besides activating the entire *vir* regulon, these molecules induce the formation of T-DNA intermediates during the process of plant infection by *Agrobacterium*. These two molecules (AS and OH-AS) are likely products of the shikimic acid biosynthetic pathway, important to a plant subjected to stress and injury.

Certain sugars induce the *vir* operon synergistically with phenolic plant metabolites by way of a distinct regulatory pathway that includes VirA and a chromosomally-encoded virulence protein, ChvE, which is a periplasmic galactose-binding protein (Cangelosi et al., 1990). Many of these sugars are known plant metabolites and monomers of major plant cell wall polysaccharides (Ankenbauer and Nester, 1990). Delmotte et al. (1991) also reported that

glycosylated phenolic inducers may act through specific sugar receptors on the bacterial cell surface.

Medium pH also plays an important role in virulence of *Agrobacterium* strains. It has been demonstrated that the induction of the *vir* genes by AS is dependent on an adaptive response of the bacterial cells to the environment; the stress put upon the bacteria by low-pH and high-osmotic-strength conditions seems to be required for the induction of these genes (Vernade et al., 1988). These authors showed that optimal induction was attained at a pH below 5.2 in a high-osmotic-strength medium containing glycine betaine; pH determined the level of *vir*G expression just as AS concentration did. Glycine betaine acts mainly by hastening the adaptation of the bacteria to a low pH, thus favoring rapid kinetics of expression of the involved genes. Starvation for phosphate also seems to partially induce the expression of the *vir*G promoter (Winans et al, 1988).

Acidic conditions are produced in nature by wounded cells. A model (Banta et al., 1994) predicts that the transmembrane protein VirA acts as an environmental sensor, mediating signal transduction upon perception of the external stimuli, mainly pH and phenolic compounds. The periplasmic domain of VirA is not absolutely required for AS-dependent *vir* induction, but it is needed for interactions with the periplasmic sugar-binding protein ChvE, which results in sugar-enhancement of phenolic

sensitivity. It has been demonstrated, in fact, that the AS receptor domain is located in the cytoplasmic domain of VirA (Turk et al., 1994).

Some practical applications of AS with or without osmoprotectants like betaine or proline, as a way of promoting higher efficiency of transformation of clover by *Agrobacterium*, are found in the literature. Several levels of AS or other phenolics and sugars have been used. Other experimental variables include medium pH, mode of application of AS, interactions of bacterial strains with plant species or cultivar, length and mode of coculture of bacterial cells with AS, and temperature.

The need for optimization of a wide range of culture conditions was clearly demonstrated by Holford et al. (1992); several variables, including medium pH, bacterial strains and levels of AS, were tested. In general, optimal transformation was favored by low pH and the inclusion of AS in the cocultivation medium, however maximal transformation of the least susceptible variety was achieved at high pH instead of low pH.

Despite all the beneficial effects of AS reported in the literature, growth inhibition and loss of virulence in cultures of *Agrobacterium* treated with AS have been observed (Fortin et al., 1992). In the course of a 6-day incubation with AS (60 μM) under acidic conditions, the authors observed up to 50% avirulent clones in some strains, whereas

control cultures not exposed to AS did not produce avirulent clones. Other *vir* inducers, sinapinic acid and syringaldehyde, also inhibited growth and promoted accumulation of avirulent strains. Furthermore, it was observed that most of the avirulent clones still carried the Ti plasmid; when present, the Ti plasmid carried the mutation leading to avirulence. A few mutants had undergone an extensive deletion of the Ti plasmid.

Mode of application, levels of acetosyringone, and pH of the induction medium

AS, the most commonly used activator of the *vir* operon, has been used mainly in two different ways, namely in liquid induction medium, or in solid cocultivation medium. A third, less common, way is to apply AS directly on the explant, prior to cocultivation with *Agrobacterium*.

Levels up to 2000 µM of AS have been studied. In a comprehensive survey of *vir*-inducers (Delmotte et al., 1991), the optimal concentration of AS was 300 µM, the same optimal concentration found by Bolton et al. (1986).

Primich-Zachwieja and Minocha (1991) tested levels ranging from 0 to 100 µM of AS, for ability to induce *Agrobacterium* activity, as measured by the induction of the *vir*E gene in a *vir*E::*lac*Z fusion plasmid. The authors found a sharp increase in activity between 0 and 20 µM AS, followed by a plateau; further experimentation, using various concentrations between 0 and 20 µM, implied a

proportionate increase in *Agrobacterium* activity, showing a concentration-dependent induction in this range. Davies et al. (1991) tested 50 µM of AS to reverse the decline in transformation of tomato explants when cocultivation with *Agrobacterium* was delayed for several hours. AS added to the induction medium maintained the competency of cells for transformation until 96 h after wounding, whereas little transformation was achieved in explants without AS.

Levels of 20 µM of AS were employed by Joao and Brown (1993), working with tomato, and Khan et al. (1994) working with subterranean clover, both with success. The rate of transformation of *Arabdopsis thaliana* Heynh.increased from ca. 3% to ca. 60% when this same level (20 µM AS) was included in the induction medium (Sheikholeslam and Weeks, 1987).

In the previously-reported experiments, AS was added directly to the liquid induction medium; however, some other experiments report the use of this compound in the solid cocultivation medium. Jacq et al. (1993) tested levels from 0 to 100 µM in MS cocultivation medium. The effects were positive only for the 75 and 100 µM treatments. The presence of 200 µM of AS in the cocultivation medium was in general beneficial for transformation of several cultivars of *Antirrhium sp.* (Holford et al., 1992). Voisey et al. (1994) supplemented their regeneration medium for white clover with 100 µM AS.

Attempts to optimize transformation efficiency of carrot root discs were made by directly pretreating the discs with two concentrations (10 and 25 µM) of AS (Guivarc'h et al., 1993). Surprisingly, while 25 µM AS applied to bacteria prior to inoculations was ineffective, the same concentration applied as a pretreatment to the discs increased the number of transformed cells and decreased the lag time to detect the appearance of the transformants. It was postulated that AS pretreatment advanced the reentry of competent cells with a potential for cell division into the S phase, and stimulated bacterial attachment to the cell walls. In this experiment, the authors also reported a synergistic effect of AS and NAA in increasing efficiency of transformation.

As stated above, acidic conditions in the induction medium are usually more favorable for the induction of the *vir* genes. Holford et al. (1992) tested pH levels of 5.2, 5.5 and 5.8, and found a negative correlation between increased pH and efficiency of transformation for most varieties of *Antirrhinum* tested. Low pH levels have also been successfully used by Joao and Brown (1993) and Kuehnle and Sugii (1991), working with tomato and *Anthurium*; pH of the induction media in these experiments was 5.4 and 5.5, respectively. The pH of non-inducing medium was maintained at 7.0 (Joao and Brown, 1993).

In an experiment carried out by Vernade et al. (1988), optimal induction was attained at a pH below 5.2 in a high-osmotic-strength medium containing glycine betaine; the authors concluded that pH determined the level of *vir*G expression just as AS concentration did.

Length of precultivation in induction medium

Jacq et al. (1993) tested cocultivation periods varying from one to six days, using 100 µM of AS applied directly in solid MS medium, in an experiment to determine factors influencing T-DNA transfer in *Agrobacterium*-mediated transformation of sugarbeet. The presence of AS permitted the reduction of the optimum infection times, and coculture for 6 days decreased transformation frequency due to bacterial overgrowth. This seems to confirm findings that a short coculture associated with AS generally produces a higher frequency of transformation. A period of 5 days of cocultivation of white clover explants in medium containing 100 µM AS was used with positive results by Voisey et al. (1994).

Cultivation of *Agrobacterium* in liquid virulence-inducing medium reported in the literature includes periods as short as (i) 5 h (James et al., 1993); (ii) 10 h, adopted by Davies et al. (1991) in their experiments, following findings by Bolton et al. (1986) that this is the optimal period; (iii) 16 h, adopted by Primich-Zachwieja and Minocha

(1991), in work trying to determine the virulence-inducing ability of exudates from 43 plants belonging to different taxa; (iv) 24 h, by Khan et al. (1994), working with subterranean clover; and (v) 48 h, adopted by Joao and Brown (1993), working with tomato.

As mentioned before, a short induction period of 5 h was used by James et al. (1993), based on the findings that after this time, the *vir*D2 products are no longer detectable. However, as stated by Winans et al. (1988), the induction kinetics for other *vir* genes is quite different, and probably some optimization has to be made.

Bacterial strains/isolates, and plant species/cultivars

The literature reports wide variation in infectivity for different strains or isolates of *Agrobacterium*, and also for several plant species or cultivars to a given strain. Wondragen et al. (1991) tested the efficiency of commonly used *A. tumefaciens* strains on 14 genotypes of chrysanthemum (*Dendrathema sp.*), and also the effect of 14 bacterial strains on the same cultivar, and found that only a few genotype/strain combinations resulted in significant tumor formation. Similar results were obtained by Barfield and Pua (1991) for *Brassica juncea* (L.) Czern. et Cosson transformation, and by Vanhala et al. (1995) for *Hyoscyamus muticus* L. transformation, and by Quesenberry et al. (1992) for red clover transformation. Jacq et al. (1993) report

that, among several hundred cultivars of sugarbeet, all were susceptible to transformation by *Agrobacterium*, although with variable frequencies..

Concluding Remarks

The preceding topics reviewed some important aspects concerning plant genetic transformation using the *Agrobacterium*-mediated transformation system. This system is being widely used in many crops, and a variety of cultivars have been released which contain transgenes for the expression of resistance to viruses and insects. It has also been shown that the ability of *A. tumefaciens* to transform plant cells can be enhanced by a variety of techniques, including the use of AS in a low-pH medium. The use of the proper type and amount of antibiotic in the selection medium was also analysed. Taken together, these points served as the basis for the experiments discussed in the following chapters.

CHAPTER 3
EFFECT OF KANAMYCIN AND GENETICIN ON
GROWTH INHIBITION IN RED CLOVER SEEDLINGS

Introduction

The use of the proper type and concentration of antibiotic in the selection medium is essential in transformation experiments, in which the antibiotic serves as the selective agent that allows only transformed cells or plants to survive. Kanamycin has been extensively used as a selective antibiotic in plant transformation experiments, mainly because chimeric genes for the expression of the Tn5 neomycin phosphotransferase II (*nptII* or *neo*) gene have become the standard selectable marker for plant transformation vectors. Geneticin traditionally has been used in animal cell cultures, for the same purpose of selection, but more recently its use has been extended to plant systems, replacing or complementing kanamycin.

Kanamycin resistance provides for the direct selection of transformants in a wide variety of plants (Rogers et al., 1987). In red clover, it has been shown that callus growth for non-transformed tissue was affected at concentrations as low as 5 $\mu g\ mL^{-1}$ kanamycin added to the tissue culture medium, and showed total inhibition of growth at

concentrations above 25 μg mL^{-1} kanamycin. Concentrations of 50 μg mL^{-1} of this antibiotic were used for selection of red clover transformants carrying the *npt*II gene (Quesenberry et al., 1992).

Geneticin, also known as antibiotic G418, has a structure which is similar to kanamycin, but has a different mode of action. It interfers with the function of the 80S ribosomes and blocking protein synthesis (Davies and Jimenez, 1980; Southern and Berg, 1982). Geneticin appears to be more potent than kanamycin in plant cells, and therefore much lower concentrations in the medium may be required.

In the present experiments, the responses of red clover seedlings to increasing concentrations of kanamycin or geneticin added to the culture medium, with or without autoclaving, were evaluated, to compare the efficacy of both antibiotics, as well as their resistance to degradation by heat during autoclaving.

Materials and Methods

Two experiments were carried out comparing concentrations of kanamycin and geneticin, with or without autoclaving (experiment 1), and increasing concentrations of geneticin (experiment 2) on the growth of red clover seedlings of the germplasm NEWRC (Quesenberry and Smith, 1993). The antibiotics were purchased from Sigma Chemical

Company (St. Louis, MO) in the form of kanamycin A monosulfate from *Streptomyces kanamyceticus*, with less than 5% kanamycin B, and geneticin disulfate salt. Stock solutions were prepared by dissolving the appropriate amount of antibiotic in distilled deionized water. The solutions were filter-sterilized using Corning disposable sterile syringe filters, 25 mm., 0.20 micron, nylon membrane (Corning Glass Works, New York, USA), dispensed in sterile screw-cap test tubes, and kept in the refrigerator.

Red clover seeds were sterilized by immersion for 5 min in concentrated sulfuric acid, washed three times with sterilized water, followed by immersion for 5 minutes in saturated sodium hypochlorite solution and washing 10 times with sterilized water. For germination, seeds were placed in 60 mm x 15 mm Petri dishes containing B5 medium (Gamborg et al., 1976) without antibiotics. Seedlings remained in B5 medium for seven days, at which time vigorous seedlings were selected, and assigned at random to the treatments. Experimental units consisted of Petri dishes containing commercial Gamborg's B5 medium from Sigma (Sigma Chemical Co. St. Louis, MO). All these procedures were conducted under sterile conditions in an EdgeGard® horizontal flow hood (Baker Co., Inc., Maine, USA).

Experiment 1 - Effect of concentrations of kanamycin or geneticin with or without autoclaving on the growth of red clover seedlings

Three concentrations of each antibiotic were tested: 25, 50 and 100 μg mL^{-1} kanamycin; and 12.5, 25 and 50 μg mL^{-1} geneticin. The antibiotics were added before autoclaving the medium, or after, when the temperature of the medium reached ca. 40 °C. A control was used in which no antibiotic was added. The experiment was carried out in four blocks, each treatment consisting of a dish containing two seedlings. The evaluations were made four weeks (blocks 1 and 2) or six weeks after initiation of the treatments (blocks 3 and 4). Blocks 1 and 2 were terminated earlier due to high rate of infection by fungi. Four variables were recorded: root length (mm) and weight (mg), shoot weight (mg), and number of leaves. Results were submitted to analysis of variance, and the means compared using Duncan's multiple range test. Data were also analyzed by means of orthogonal contrasts. All statistical analyses used the statistical analysis package (SAS, 1985).

Experiment 2 - Effect of concentrations of geneticin on the growth of red clover seedlings

Eight concentrations of geneticin were tested: 0, 3, 6, 9, 12, 15, 18, and 21 μg mL^{-1}, added after autoclaving. The experiment was carried out in three blocks, each treatment consisting of a dish containing three red clover seedlings.

Seven variables were recorded six weeks after initiation of the treatments: root length (mm) and weight (mg), crown weight (mg), leaf number, green leaf percentage (visual estimate) and weight (mg), and dead leaf weight (mg); leaf weights included the leaf petioles. For the purpose of this experiment, crown was considered as the thickened transition zone between root and leaves, which could not be classified in either of the two. Results were submitted to analysis of variance, and the means were compared using the same test as described above for experiment 1.

Results

Experiment 1 - Effect of concentrations of kanamycin or geneticin with or without autoclaving on the growth of red clover seedlings

The analysis of variance for kanamycin and geneticin together showed significant differences between treatments for all variables, and for blocks for the number of leaves (Table 3-1); blocks 1 and 2, which were evaluated earlier, had a lower number of leaves, on average (data not presented). The means are presented in Table 3-2. In general, geneticin at the same concentrations as kanamycin proved to be much more toxic. Root and shoot weights were significantly higher in the control, compared to all other treatments. However, root length was significantly higher for the 25 $\mu g\ mL^{-1}$ kanamycin autoclaved treatment, compared

to all other treatments. The number of leaves did not differ from the control and in the treatments that received 25 μg mL^{-1} kanamycin with or without autoclaving, or 50 μg mL^{-1} autoclaved, but these treatments were significantly different from the remaining treatments. Fig. 3-1 and 3-2 present the results for kanamycin and geneticin, respectively, as a percentage of the control seedlings.

The effect of autoclaving on the stability of the antibiotics was tested separately for kanamycin and geneticin. Table 3-3 lists mean square values for the contrasts among the treatments within each antibiotic. Significant differences are shown for several contrasts for root length and number of leaves in the kanamycin treatments, and for number of leaves in the geneticin treatments.

Root length and number of leaves tended to be higher when kanamycin was autoclaved, indicating deactivation of the antibiotic during the autoclaving. Number of leaves for the autoclaved treatments of geneticin, however, tended to be lower, an effect opposite to that observed for kanamycin; on average, autoclaving geneticin reduced the number of leaves/seedling by 30% (Table 3-2). These effects are depicted in Fig. 3-3, which shows the efficacy of the antibiotics after autoclaving, as a percent of the non-autoclaved treatment responses.

Table 3-3 also presents results for the orthogonal contrasts comparing the control (no antibiotics added) to the treatments that received antibiotics, either kanamycin or geneticin. Treatments receiving geneticin were significantly different from the control for all variables analyzed, whereas, for kanamycin, root length did not differ among treatments and control.

Experiment 2 - Effect of concentrations of geneticin on the growth of red clover seedlings

Differences among the geneticin concentrations were found for all variables analyzed with the exception of dead leaf weight (Tables 3-4 and 3-5). Furthermore, root length and weight, number of leaves, and green leaf weight showed significant differences among the control and all other treatments.

Discussion

The effects of kanamycin, geneticin and other aminoglycoside antibiotics vary, depending upon several factors, including concentration and plant genotype. For a given protocol, kanamycin is usually less toxic than geneticin for plant cells. For instance, Catlin (1990) found that sugarbeet callus growth was inhibited by levels of 50 $\mu g\ mL^{-1}$ geneticin, as opposed to 150 $\mu g\ mL^{-1}$ kanamycin; other aminoglycoside antibiotics tested by this author were gentamicin, hygromycin, and phleomycin, with the following

inhibitory doses: 150 μg mL^{-1}, 10 μg mL^{-1}, and 20 μg mL^{-1}, respectively. In the same experiment, total inhibition of shoot formation from callus was achieved at kanamycin concentrations as high as 150 μg mL^{-1}, whereas 10 μg mL^{-1} geneticin was able to completely inhibit shoot formation.

For red clover, kanamycin has been used when the transformation vector contains the *npt*II gene. The first experiment reported in this chapter attempted to explore the possible use of geneticin in this species. Two main conclusions can be drawn from that experiment, in terms of the type and concentrations of antibiotics used. First, 50 μg mL^{-1} kanamycin provided a sufficient level of toxicity to hinder development of clover seedlings. Although it did not kill the seedlings, their biomass accumulation was greatly reduced when kanamycin was present in the medium. This corroborates results previously reported (Quesenberry et al., 1992) that low concentrations of kanamycin are toxic to red clover, inhibiting callus formation in red clover explants. The question still remains as to whether the responses of red clover calli and seedlings to kanamycin differ.

It is also important to realize that plant genotype is an important factor in resistance to antibiotics. Norelli and Alswinckle (1993) found a broad spectrum of tolerance of several apple genotypes to aminoglycoside antibiotics, some genotypes being 38 times more resistant to paromomycin, 2.5

times to geneticin, and 3.8 times to neomycin. A broad, unexplained, variability among different experiments was found by these authors. In one experiment, regeneration was inhibited at 10 $\mu g\ mL^{-1}$ kanamycin, but in another experiment, regeneration was not inhibited until a concentration of 100 $\mu g\ mL^{-1}$ kanamycin was used.

It is also important to take into consideration that kanamycin in plant tissue culture medium is probably deactivated with time, and plants remaining on the same medium for a prolonged period may develop normaly, even without being resistant to kanamycin.

Another important conclusion is that geneticin can provide a suitable alternative for use in the selection medium at lower concentrations than kanamycin. In fact, all concentrations of geneticin used in the experiment proved highly toxic to seedlings, as shown not only in reduction of biomass accumulation, but also in decrease in root elongation and in the number of leaves.

Most of the information in the literature points to a greater toxicity for geneticin, when compared to kanamycin. Catlin (1990) observed that similar effects on sugarbeet were obtained with geneticin concentrations 5 to 15 times lower than kanamycin. Norelli and Aldwinckle (1993) observed complete inhibition of apple callus formation by 1 $\mu g\ mL^{-1}$ geneticin, as compared to 5 $\mu g\ mL^{-1}$ kanamycin. Tsang et al. (1989) tested the toxicity of various aminoglycoside

antibiotics on zygotic embryos of *Picea glauca*, and found that total inhibition of bud formation was achieved by 10 µg mL^{-1} geneticin, whereas 20 to 50 µg mL^{-1} kanamycin concentrations were needed for total inhibition. Rice has also a much greater sensitivity to geneticin than to kanamycin (Dekeyser et al., 1989). In a few cases, however, kanamycin and geneticin had virtually the same effect. Orlikowska et al. (1995) found that concentrations of 75 µg mL^{-1} geneticin and 60 µg mL^{-1} kanamycin had the same inibitory effect on safflower callus formation.

When examined in greater detail, the results also reveal that two of the variables analyzed, root and shoot weight, have a higher discriminatory power among treatments than root length or number of leaves. Observations made during the course of the experiment revealed that roots in some treatments, particularly those that received 25 µg mL^{-1} kanamycin (autoclaved or not) and 50 µg mL^{-1} autoclaved, were long but thin and unbranched. This accounts for the fact that, although root weight was statistically different among these treatments and the control, root length was not, hindering the value of this response-variable in experiments assessing the efficacy of antibiotics in plants, which is associated with the possible growth regulator-like effect of antibiotics, as discussed below.

Addition of components such as antibiotics to medium after autoclaving is a time-consuming operation,

particularly when large volumes of medium are required, because the medium must be allowed to cool down to a precise temperature, close to 40 °C, low enough to not deactivate the added compounds, but high enough to mainatain a liquid medium. The results obtained in the comparisons between autoclaved and non-autoclaved treatments reveal that some deactivation of kanamycin was caused by its addition before autoclaving the medium, as can be seen by the greater root length and number of leaves, whereas geneticin was not deactivated. In fact, the number of leaves in the autoclaved geneticin treatments was significantly lower than in the non-autoclaved treatments with the same antibiotic.

It is difficult to formulate hypotheses when confronted with apparently contradictory results. One could postulate that autoclaving kanamycin simply decreased the efficiency of this antibiotic, and therefore the roots were able to elongate more compared to the non-autoclaved treatment. However, the roots were in fact longer than the roots in the control treatments, which did not receive any antibiotic. This could be due to the release of some chemical with growth regulator-like activity during the autoclaving.

Some antibiotics have been shown to promote growth or embryogenesis in plant tissue culture. For instance, Moore et al. (1988) found that methotrexate and chloramphenicol induced the formation of embryogenic callus in 'Key' lime. Norelli and Aldwinckle (1993) tested the effects of several

concentrations of aminoglycoside antibiotics in the regeneration and selection of transgenic apple tissue expressing the *npt*II gene, and observed that paromomycin and neomycin increased the percent regeneration at doses below the inhibitory concentration, whereas kanamycin and geneticin did not. Cefotaxime and carbenicillin, ß-lactam antibiotics that inhibit cell wall synthesis in bacteria, and are used to eliminate *Agrobacterium* in transformation experiments, have also been found to promote apple regeneration (James et al., 1990).

Carbenicillin and penicillin G also have been shown to produce physiologically active levels of phenylacetic acid, an auxin shown to stimulate callus formation and growth of *Anthirrium majus* (Holford and Newbury, 1992). Catlin (1990) observed similar effects for kanamycin and phleomycin. Relatively low levels of kanamycin increased the number of shoots recovered from sugarbeet tissue culture, whereas it had almost no effect on the number of explants forming callus. Geneticin tested in the same experiment had no positive effect on growth, and totally inhibited shoot formation at concentrations of 10 $\mu g\ mL^{-1}$. Yepes and Aldwinckle (1994a) observed that cefotaxime at 250 $\mu g\ mL^{-1}$ enhanced regeneration and shoot development in apple explants, and carbenicillin at 500 $\mu g\ mL^{-1}$ induced abundant callus formation but inibited regeneration. In another experiment (Yepes and Aldwinckle, 1994b), cefotaxime at 200

$\mu g\ mL^{-1}$ stimulated shoot growth and development, but at 500 $\mu g\ mL^{-1}$ caused abnormal shoot morphology. Carbenicillin at 500 $\mu g\ mL^{-1}$, alone or with 200 $\mu g\ mL^{-1}$ cefotaxime, inhibited proliferation, caused callus formation, and the release of phenolics into medium. Cefotaxime also showed positive effects in regeneration of cereals (Mathias and Boyd, 1986; Mathias and Mukasa, 1987).

The concentrations of geneticin chosen for the first experiment were tentative, based solely on the established fact that geneticin is generally more toxic than kanamycin. The results of experiment 1 reveal that even the lower concentration, 12.5 $\mu g\ mL^{-1}$ of geneticin, was extremely toxic to red clover. The second experiment was conducted to analyze in greater detail the effects of geneticin on the growth of red clover seedlings. Lower concentrations of the antibiotic were chosen, and some additional variables were recorded. Most of the variables analyzed, with the exception of dead leaf weight, were useful in the discrimination of treatments, as seen in the results from the analysis of variance.

Very low concentrations of geneticin were employed in this experiment, ranging from as low as 3 $\mu g\ mL^{-1}$, and the results from the analysis of variance show that, for most variables, the controls were significantly different from all treatments, indicating that the toxicity of geneticin to red clover seedlings is more than ten-fold that of

kanamycin. Further experimentation would be desirable on the effects of lower concentrations of geneticin on red clover, including the effects on callus formation.

Furthermore, geneticin was not deactivated by heat during autoclaving, which may constitute a great advantage since it may be added to the medium before autoclaving, greatly facilitating the process of medium preparation in a tissue culture laboratory.

Table 3-1. Mean squares from the analyses of variance for root length and weight, number of leaves, and shoot weight, for red clover seedlings cultivated in B5 medium containing kanamycin or geneticin (treatment). Control seedlings were grown on similar medium without addition of antibiotics.

Source of variation	df	Length	Weight	Number of leaves	Shoot weight
Block	3	926.5	2959.2	5.6**	8086.0
Treatment	12	17670.8**	117880.6**	9.0**	124061.2**
Error	36	732.3	4371.5	0.7	5541.4

** F test significant at the 1% level.

Table 3-2. Effect of kanamycin or geneticin on growth of red clover. Seedlings were cultivated in B5 medium containing several concentrations of kanamycin or geneticin, indicated in $\mu g\ mL^{-1}$, and data recorded four to six weeks after exposure to kanamycin or geneticin. The antibiotics were added to the medium before autoclaving (Aut.) or after (N/aut.). Control seedlings were grown on similar medium without the addition of antibiotics.

Treatment	Root		Number of leaves	Shoot weight
	Length	Weight		
	------- mg ---------		--number--	---mg---
Control	134b	632a	6.0a	680a†
Kanamycin				
25-Aut.	246a	178b	5.9a	259b
25-N/aut.	136b	102bc	5.0a	172bc
50-Aut.	111b	64c	5.5a	167bc
50-N/aut.	48c	29c	3.8b	108cd
100-Aut.	34c	29c	3.5bc	63cd
100-N/aut.	48c	18c	3.7b	108cd
Geneticin				
12-Aut.	30c	7c	2.2cde	51cd
12-N/aut.	31c	13c	3.1bcd	62cd
25-Aut.	33c	10c	1.7e	37d
25-N/aut.	28c	9c	2.7bcde	42d
50-Aut.	29c	10c	2.0de	39d
50-N/aut.	26c	6c	2.2cde	28d

† Means with the same letter within columns do not differ at the 5% level (Duncan's multiple range test).

Table 3-3. Mean squares from the analyses of variance for the orthogonal contrasts for root length, root fresh weight, number of leaves, and shoot weight, for red clover seedlings cultivated in B5 medium containing kanamycin (K) or geneticin (G). Each antibiotic was applied at three concentrations, indicated after the antibiotic code, in $\mu g\ mL^{-1}$, with or without autoclaving (Aut and N/a, respectively). Control seedlings were grown on similar medium without the addition of antibiotics.

Source of variation	df	Root		Number of leaves	Shoot weight
		Length	Weight		
Kanamycin					
Aut vs N/a	1	16748**	9801	3.7*	6384
K25-Aut vs N/a	1	24200**	11626	1.5	15138
K50-Aut vs N/a	1	7938*	2346	6.1**	7021
K100-Aut vs N/a	1	420	231	0.1	4050
K25 vs K50	1	49729**	34969*	2.6	23947
K25 vs K100	1	89700**	54056*	13.1**	67081*
K50 vs K100	1	5852*	2070	4.0*	10868
Control vs K concentr.	1	3102	1082091**	7.1**	977525**
Error	18	1172	8173	0.7	10160
Geneticin					
Aut vs N/a	1	37	2	3.0**	51
G12-Aut vs N/a	1	3	85	1.5**	276
G25-Aut vs N/a	1	50	0	2.0**	128
G50-Aut vs N/a	1	28	30	0.1	242
G12 vs G25	1	0	1	0.8	945
G12 vs G50	1	42	8	1.3*	2139
G25 vs G50	1	45	3	0.1	240
Control vs G concentr.	1	37202**	1329322**	45.6**	1389934**
Error	18	241	7229	0.2	8273

*, ** F test significant at the 5% and 1% levels, respectively.

Table 3-4. Mean squares from the analyses of variance for root length and weight, crown weight, number of leaves, green leaf percentage and weight, and dead leaf weight, for red clover seedlings cultivated in B5 medium containing several concentrations (Conc.) of geneticin.

Source	df	Root		Crown weight	Number of leaves	Green leaf		Dead leaf weight
		Length	Weight			%	Weight	
Conc.(C)	7	229**	139**	25.4**	4.0**	26.5**	451**	19†
Block(B)	2	1	109**	16.1**	0.0	5.1	125*	23
C X B	14	1	72**	5.4*	0.1	5.0	64*	8
Error	47	5	13	2.6	0.2	4.8	31	14

*, ** F test significant at the 5% and 1% level, respectively.
† Observed values = tabulated values x 10^2.

Table 3-5. Effect of geneticin on growth of red clover. Seedling were cultivated in B5 medium containing several concentrations (Conc.) of geneticin, indicated in $\mu g\ mL^{-1}$. Data was recorded six weeks after exposure to the antibiotic.

Conc.	Root		Crown weight	Number of leaves	Green leaf		Dead leaf weight
	Length	Weight			%	Weight	
	--mm--	---- mg	-----			---- mg	-----
0	200a	334a	57a	10.1a	56a	215a	83a†
3	49b	52b	32bc	6.4b	42ab	60b	84a
6	48b	20bc	31bc	5.6bc	33bc	43b	80a
9	51b	16bc	35b	5.5bc	26bcd	31b	63a
12	52b	9c	42ab	5.3bc	35abc	48b	74a
15	48b	11c	18cd	4.4cd	19cd	13b	63a
18	52b	7c	10d	3.5d	10d	4b	50a
21	42b	5c	9c	3.2d	5d	3b	45a

† Means with the same letter in the column do not differ the 5% level (Duncan's multiple range test).

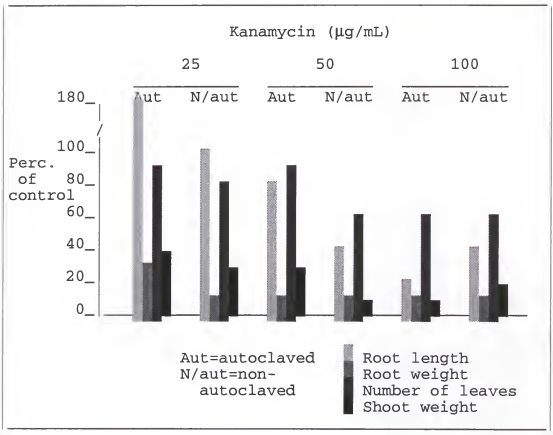

Fig. 3-1. Effect of the concentration of kanamycin, with or without autoclaving, on the growth of red clover seedlings, as a percent of control (Perc. of control; control= seedlings growing in the same medium without the addition of kanamycin). **Methods**: seedlings were plated on B5 medium containing kanamycin at the concentrations indicated, where they remained for four to six weeks. Kanamycin was added before autoclaving the medium (Aut), or after, when the medium temperature had reached 40 °C (N/aut).

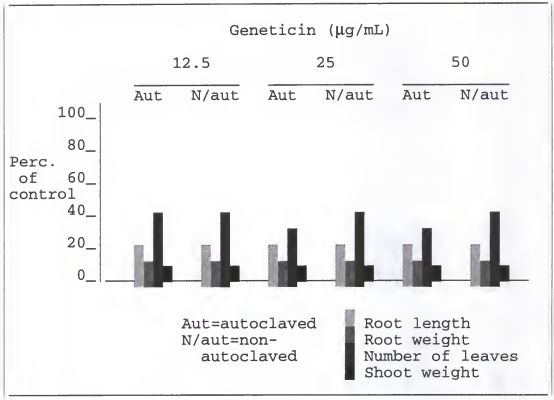

Fig. 3-2. Effect of the concentration of geneticin, with or without autoclaving, on the growth of red clover seedlings, as a percent of control (Perc. of control; control= seedlings growing in the same medium without the addition of geneticin). **Methods**: seedlings were plated on B5 medium containing geneticin at the concentrations stated, where they remained for four to six weeks. Geneticin was added before autoclaving the medium (Aut), or after, when the medium temperature had reached 40 °C (N/aut).

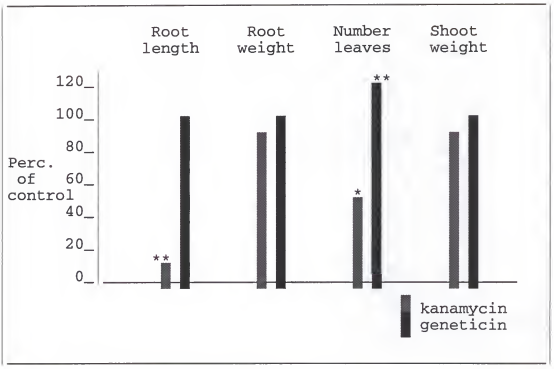

Fig. 3-3. Effect of autoclaving on the toxicity of kanamycin and geneticin to red clover seedlings, as a percent of the toxicity of the control treatment within each group (Perc. of control; control= seedlings growing in the same medium without the addition of antibiotic). **Methods**: seven-day old seedlings were plated on B5 medium containing kanamycin or geneticin at three concentrations, where they remained for six weeks. The symbols *, ** denote significant differences among autoclaved and non-autoclaved treatments within each antibiotic group, according to the F test at the 5% and 1% levels, respectively.

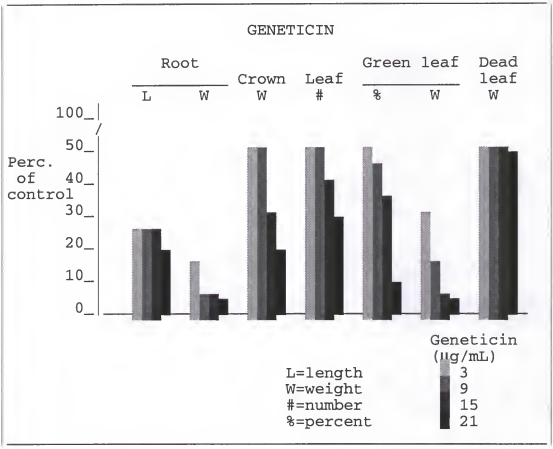

Fig. 3-4. Effect of concentration of geneticin on the growth of red clover seedlings, as a percent of control (Perc. of control; control= seedlings growing on the same medium without geneticin). **Methods**: seven-day old seedlings were plated on B5 medium containing geneticin at the concentrations stated, where they remained for six weeks.

CHAPTER 4
EFFECT OF ACETOSYRINGONE ON THE GROWTH
AND VIRULENCE OF *AGROBACTERIUM TUMEFACIENS* STRAINS

Introduction

Several factors can influence the virulence of *Agrobacterium* strains, which in turn will determine the efficiency of plant transformation in experiments involving *Agrobacterium*-mediated DNA transfer. Acetosyringone (AS; 3'5'-dimethoxy-4'hydroxyacetophane) has been implicated as an important phenolic compound for the induction of virulence in *Agrobacterium tumefaciens*. This compound interacts with the Ti-plasmid genes *vir*A, *vir*G and *vir*F (Palmer and Shaw, 1992). Other compounds, including synthetic AS, acetovanillone, syringaldehyde, and syringic acid beta-glycosides, have also been shown to induce the *vir* operon (Delmotte et al., 1991).

Another important factor in *Agrobacterium* virulence is medium pH (Joao and Brown, 1993; Vernade et al., 1988). Although *Agrobacterium* is able to grow in media with pH ranging from 4.5 to 11.0, with an optimal at 7.25 (Bell et al., 1990), Holford et al. (1992) found a negative correlation between pH and efficiency of transformation of

several varieties of *Antirrhinum*, when the bacteria grew on media with pH levels of 5.2, 5.5 and 5.8.

The literature also reports wide variation in infectivity for different strains or isolates of *Agrobacterium*, and for the response of plant species or cultivars to a given strain. For example, only a few genotype/strain combinations resulted in significant tumor formation when several genotypes of chrysanthemum and several strains of *A. tumefaciens* were tested (Wondragen et al., 1991). Similar results were obtained by other authors working with different plant species (see, eg., Jacq et al., 1993; Vanhala et al., 1995).

Considering the influence of these many factors, experiments were carried out to determine the effect of acetosyringone and medium pH on the growth kinetics and on the ability of several strains or isolates of *A. tumefaciens* to transform explants of red clover and tobacco.

Materials and Methods

Tobacco (*Nicotiana tabacum* L.) cv. Xanthi, and red clover (*Trifolium pratense* L.) germplasm NEWRC (Smith and Quesenberry, 1995), and several strains and isolates of *A. tumefaciens* and *Escherichia coli* were used in the experiments. Stock solutions of AS (Aldrich Chemical Co., 97% purity) were prepared by dissolving the appropriate amount in 95% ethanol. The solutions were filter-sterilized

prior to using. YEP medium (10 g yeast extract, 10 g peptone and 5 g sodium chloride per liter) was used for all liquid bacterial cultures.

The *A. tumefaciens* and *E. coli* strains and plasmids used in the experiments are listed in Table 4-1. Strain EHA101 (Hood et al., 1986; Lulsdorf et al., 1991) has a C-58 chromosomal background, and contains the disarmed plasmid pEHA101, derived from the octopine plasmid pTiBo542 obtained from strain A281 by deletion of the T-DNA sequences. Binary transformation vector plasmids pMON9793 and pMPSV4-43, described in Chapter 5, were used in conjunction with EHA101. The plasmid pMPSV4-43 was mobilized into this strain by direct DNA uptake; several transformed colonies were isolated (see Chapter 5), and colonies 1, 11, 15, 20, 28, 29 and 30 were used in several experiments. Strain Z707 (Hepburn et al., 1985) contains the plasmid pZ707, a derivative of nopaline-type pTiC58, in a nalidixic-acid resistant C-58 chromosomal background. Strain LBA4404 contains the plasmid pAL4404, which is a T-DNA deletion of the octopine-type pTiAch5; this strain is derived from LBA4213(pAL231) (Jen and Chilton, 1986; Ooms et al., 1982). The vector plasmid pBI121 (Jefferson et al., 1987), containing a mutated *npt*II gene from transposon *Tn*5 (Yenofsky et al., 1990) and the ß-glucuronidase (*gus*) gene from *E. coli*, was tested in this strain. Strain T37 contains the wild-type nopaline plasmid pTiT37, in addition to the

binary plasmid pMON9793. Finally, A281 is a derivative of A136 by *in planta* conjugation with strain Bo542 (Sciaki et al., 1978), which resulted in a wild-type octopine strain with a C58 chromosomal background. The binary plasmid pGA482GG (An, 1995), which contains a wild-type *npt*II gene and both left and right T-DNA borders, was used with this strain.

Experiment 4-1. Effect of 50 µM AS or 10 mM ethanol on the growth of *A. tumefaciens* and *E. coli*

In order to detect any detrimental effect of AS or alcohol, which was used to prepare the AS stock solutions, on bacterial growth, an experiment was designed using several strains or isolates of these two bacterial species (Table 4-3). Bacteria were grown in the presence of 50 µM AS, in liquid YEP induction medium, pH 5.2, and in YEP pH 7.0, as normally used in our laboratory to grow *Agrobacterium*. As a control, the same material was grown in YEP media (pH 5.2 and 7.0) without AS, or on the same media with the addition of an amount of ethanol equivalent to the amount added from the stock solution of AS.

The acidic medium was prepared following Ankenbauer and Nester (1990), with buffering capacity being provided by 0.5 mM monobasic phosphate buffer and 50 mM MES [2-(N-morpholino) ethanesulfonic acid], both added directly to the medium before autoclaving. Cultures of *A. tumefaciens* and *E. coli* were grown overnight in YEP medium pH 7.0, with the

appropriate amounts of antibiotics according to the requirements for each strain. Optical density readings at 600 nm (OD_{600}) were taken, cultures were centrifuged at 4,200 rpm for 5 min, and then resuspended in the appropriate amount of YEP, pH 7.0, to achieve a density of 5×10^8 colony-forming units (CFU) mL^{-1}. Aliquots (100 µL) were drawn and added to 12-mL culture tubes (Stardest) containing 5 mL of the appropriate medium, according to treatment, and incubated in a rotary shaker at 240 rpm and 28 °C. One mL aliquots were drawn after 12 and 36 h of incubation for measurement of the OD_{600}, to estimate growth. Medium pH was also evaluated after 36 h of growth. The experiment was carried out in three replicates in a completely randomized design. Data were subjected to analysis of variance, and the means compared using Duncan's multiple range test. Data were also subjected to cluster analysis using the complete linkage method, with data standardized to mean zero and variance one.

Experiment 4-2. Effect of increasing concentrations of AS (0, 5, 10, 20, 40, 80, 160 and 320 µM) and medium pH on the growth of *A. tumefaciens*

Six strains or isolates of *A. tumefaciens* were grown in increasing concentrations of AS, in YEP medium at two pH levels (5.2 and 7.0). The *Agrobacterium* strains used were A281(pGA482GG), EHA101(pMSV4-43/20), EHA101(pMPSV4-43/30), LBA4404(pBI121), T37(pMON9793), and EHA101(pMON9793). The

same protocol as in experiment 4-1 was followed for the growth of *Agrobacterium*. Estimation of growth was performed 12 h after inoculation of the medium. , from OD_{600}. Data were analyzed using standard analysis of variance techniques.

Experiment 4-3. Effect of increasing concentrations of AS (0, 20, 80 and 320 µM) and medium pH on the growth of *A. tumefaciens*

Several strains or isolates of *A. tumefaciens* were grown in the absence of AS or in the presence of increasing concentrations of this compound, in YEP medium at two pH levels: 5.2 and 7.0. The strains of *Agrobacterium* used and the experimental protocol are the same listed for the previous experiment. Estimation of growth (OD_{600}) was done 36 and 72 h after inoculation of the medium. The data were analyzed using analysis of variance.

Experiment 4-4. Effect of medium pH on callus weight in red clover explants

The effects of YEP medium at pH 5.2 and 7.0 on growth of red clover callus was evaluated. The explants were dipped in liquid YEP medium and immediately plated on B5 callus medium (pH 5.8), five explants per experimental unit. Callus weights were recorded 12 wk later, during which time cultures were maintained in a Percival growth chamber (Percival, Boone, Iowa) with controlled humidity, at 28 °C and 40 µmol m^{-2} s^{-1} of light from fluorescent lamps, with a

photoperiod of 16 h. Callus weight data were analyzed using analysis of variance.

Experiment 4-5. Effect of increasing concentrations of AS (0, 80 and 320 µM) and medium pH on the virulence of *A. tumefaciens* in red clover and tobacco

Three concentrations of AS were tested on four *Agrobacterium* strains: A218(pGA482GG), EHA101(pPSV4-43/20), LBA4404(pBI121), and EHA101(pMON9793), in acidic medium (pH 5.2). Additionally, the same concentrations of AS were tested on strain LBA4404(pBI121) at two pH levels: acidic (pH 5.2) and neutral (pH 7.0). *Agrobacterium* was grown overnight in liquid YEP medium, pH 7.0. The OD_{600} was determined, cultures were centrifuged at 4,000 rpm for 10 min and resuspended in liquid induction medium, as described above, to a density of 5×10^8 CFU mL^{-1}. The cell suspensions were allowed to grow for an induction period of 10 h. Red clover petiole segments and tobacco leaf discs were cocultivated with bacterial suspensions following protocols described in Chapter 5, for a period of 48 h, and then transferred to the appropriate medium, containing 50 µg mL^{-1} kanamycin (red clover) or 100 µg mL^{-1} kanamycin (tobacco), for selection of transformants, and 300 µg mL^{-1} carbenicillin, for elimination of *Agrobacterium*. Virulence was assessed by callus weight 35 days (d) after cocultivation for red clover and tobacco, and by number of shoots formed at the same period, for tobacco only.

Results

Experiment 4-1. Effect of 50 µM AS or 10 mM ethanol on the growth of *A. tumefaciens* and *E. coli*

Analyses of variance results (Table 4-2) indicated significant differences among strains and between pH levels for all variables. Significant interactions strain x medium for bacterial growth at 12 h, and strain x final medium pH at 36 h, were also detected. The addition of either 50 µM of AS or alcohol in the same amount used to dissolve the AS did not have any effect on bacterial growth or on final medium pH. On the other hand, the initial pH of the YEP medium had a marked effect on bacterial growth, with neutral medium being more conducive to growth (Table 4-3).

The trends for *A. tumefaciens* strains T37(pMON9793) and Z707(pMPSV4-43), and, to some extent, LBA4404, were higher OD_{600} and lower final medium pH than the other strains (Table 4-3). The strains LBA4404 and T37(pMON9793) were further characterized by presenting the lowest and the highest differences between the final medium pH on neutral and acidic medium (0.8 and 1.5 pH units, respectively). Strain LBA4404 increased the pH of the acidic medium (from 5.2 to 5.7), and, at the same time, decreased the pH of the neutral medium (from pH 7.0 to pH 6.5), whereas strain T37(pMON9793) produced smaller changes in medium pH.

E. coli strain HB101(pMON9793) had the lowest OD_{600} at both 12 and 36 h, and also low final medium pH values in

both media. *E. coli* strain mm294(pMON9793) had equally low values for OD_{600} at 12h, and for the final medium pH. All the remaining strains were characterized by moderate growth, little or no change in pH on the neutral medium, and some pH increase of the acidic medium (Table 4-3).

The results from the cluster analysis are presented in Fig. 4-1. *A. tumefaciens* strains T37(pMON9793) and Z707(pMPSV4-43) formed a cluster based on high OD_{600} and low final medium pH, as discussed earlier. *E. coli* strains HB101(pMON9793) and mm294(pMON9793) had, in general, low values for OD_{600} and final medium pH, and are clustered together at the opposite end of the dendrogram. Strain LBA4404, although also with high OD, has low and high pH, respectively for the neutral and acidic media, and formed a cluster by itself. The remaining *A. tumefaciens* strains (EHA101) were clustered together, by virtue of the moderate values for OD, high pH on neutral medium, and intermediate pH on the acidic medium, as discussed earlier.

Experiment 4-2. Effect of increasing concentrations of AS (0, 5, 10, 20, 40, 80, 160 and 320 µM) and medium pH on the growth of *A. tumefaciens*

The analyses of variance revealed differences for strain, medium and concentration of AS for bacterial growth 12 h after inoculation (Table 4-4). The strain x medium interaction was primarily due to the fact that strain T37(pMON9793) had higher growth than strain EHA101(pMON9793)

on medium pH 7.0, with an opposite response observed on medium pH 5.2. Comparison of the growth of the *Agrobacterium* strains showed that strain T37(pMON9793) had the highest OD_{600} values, and strain A281(pGA482GG) the lowest; on average, the former grew twice as much. The remaining strains were intermediate in growth (Table 4-5).

Medium pH had a marked effect on bacterial growth. Neutral conditions were much more conducive to *Agrobacterium* growth than acidic conditions; growth was almost three fold on medium pH 7.0, compared to growth on medium pH 5.2.

High concentrations of AS caused a delay in the growth of *Agrobacterium*, as can be seen by the significantly lower OD_{600} values when bacteria were grown in medium containing AS concentrations of 80 µM and above (Table 4-5). AS concentration, however, did not influence final medium pH. Contrary to the results of the previous experiment, there was a marked increase in the final medium pH in both acidic and neutral media.

Experiment 4-3. Effect of increasing concentrations of AS (0, 20, 80 and 320 µM) and medium pH on the growth of *A. tumefaciens*

The analyses of variance revealed differences for strain, medium pH, concentration of AS, and for the interaction strain-medium pH, for the response variable bacterial growth 36 h after inoculation, and for strain-

medium pH for the response variable bacterial growth 72 h after inoculation (Table 4-6).

Strain T37(pMON9793) had the highest OD_{600} values, and strain LBA4404(pBI121) the lowest at 36 and 72 h (Table 4-7). The remaining strains were intermediate in growth. Two isolates of strain EHA101(pMPSV4-43) -isolates 20 and 30 - showed significant differences in growth 36 h after inoculation.

Acidity also had a marked effect on bacterial growth. At 36 h after inoculation, neutral conditions were more conducive to growth, whereas 72 h after inoculation, this response was reversed (Table 4-7). The differences at both periods were approximately 0.1 OD unit, considerably lower than the differences observed at 12 h in the previous experiment.

High concentrations of AS seemed to cause delay in the growth of *Agrobacterium*, as can be seen by the lower OD_{600} values at 36 h, when bacteria grew in media containing 320 µM; however, at 72 h, the AS effect on growth had ceased (Table 4-7).

Figs. 4-2 and 4-3 combine the results from the experiments testing the effects of AS concentration and medium pH on *Agrobacterium* growth. They clearly illustrate the same tendency, namely that both AS concentration and acidic medium hinder bacterial growth during the initial

phases of growth, but the effects are no longer observed at 72 hours.

Experiment 4-4. Effect of medium pH on callus weight in red clover explants

There were significant differences between the two media (Table 4-8); acidic conditions in the medium decreased callus weight in red clover from 192.5 mg in pH 7.0 to 151.7 mg in pH 5.2 (Table 4-9).

Experiment 4-5. Effect of increasing concentrations of AS (0, 80 and 320 µM) and medium pH on the virulence of *A. tumefaciens* in red clover and tobacco

The analyses revealed differences for AS concentration in both species, and additionally for medium pH in red clover (Table 4-10). For red clover, the mean callus weight on pH 5.2 medium (66.6 mg) was significantly higher than the weight on pH 7.0 medium (31.5 mg)(Table 4-11). Callus weight was also significantly higher in AS concentrations 80 and 320 µM (70.4 mg).

For tobacco, there were no signifficant differences between pH levels for callus weight and number of shoots. However, a concentration of 80 µM AS produced significantly higher callus weight and number of shoots than the 0 AS concentration. The highest concentration of AS (320 µM) gave intermediate results (Table 4-11).

The virulence of four strains of *Agrobacterium* was assessed on red clover explants on medium at pH 5.2. The

analysis of variance (Table 4-12) revealed significant differences among strains. Strains EHA101(pMPSV4-43/20), LBA4404(pBI121) and A281(pGA482GG) promoted significantly higher callus growth compared to strain EHA101(pMON9793) (Table 4-13). The concentration of AS, however, did not seem to have any effect on callus weight when these four strains of *Agrobacterium* were considered (Table 4-13).

Fig. 4-4 and 4-5 combine together the results from the experiments testing the influence of AS concentration and medium pH on *A. tumefaciens* virulence, based on the rate of callus formation in tobacco and red clover explants.

Discussion

In the experiments reported here, the effects of medium pH, strain, and AS concentrations on the growth of *A. tumefaciens* and *E. coli*, and on the ability of *A. tumefaciens* to transform red clover and tobacco explants were studied.

Effect of pH

Acidic conditions in the medium have been reported to reduce bacterial growth (Fortin et al., 1992). Bell et al. (1990) observed that the optimum pH for growth of strains of *Agrobacterium* was 7.25, and reduced growth was observed at extreme pH values (4.5 and 11.0).

In the present experiments, medium acidity appeared to only affect growth during the initial phases of growth of *E. coli* and *A. tumefaciens*. Measurements taken 12 h after inoculation showed severe reduction in growth, up to three-fold, when acidic and neutral media were compared. The negative effects of pH decreased by 36 h after inoculation, and were reversed 72 h after inoculation (Tables 4-3, 4-5 and 4-7). Fortin et al. (1992) also observed that at early stages the growth of *A. tumefaciens* was retarded due to AS, but at later stages (144 h after inoculation), no effects were apparent. Furthermore, the authors noted that the growth of octopine-type strains was less affected by AS than the growth of nopaline strains.

Dion et al. (1995) found that the growth retardation due to AS is more pronounced at lower pH (e.g., 5.3 and 5.6), compared to pH 5.8, and that no significant effect can be observed above pH 5.8. The results of our experiments, however, showed a significant delay of growth at both pH 5.2 and 7.0, when high levels of AS are used in the growth medium.

It has also been shown that optimal induction of the virulence genes can be attained at low pH (Vernade et al., 1988; Holford et al., 1992). In the present experiments, pH 5.2 significantly increased the apparent virulence of the octopine-type strain LBA4404(pBI121) on red clover (Table 4-11). However, medium pH had no effect on the virulence of

this strain when tested with tobacco explants (Table 4-11). This could indicate that red clover is less likely than tobacco to induce the *vir* genes in *Agrobacterium*. Tobacco seemed to be indifferent to medium acidity, and this may indicate that this plant species has the ability to induce comparable virulence in neutral and acidic medium.

Acidic medium negativelly affected callus formation in red clover in the absence of both AS and *A. tumefaciens*, since callus weight from red clover explants exposed to YEP medium pH 5.2 were significantly lower as compared to medium pH 7.0 (Table 4-9), even though the pH of B5 medium, utilized for callus formation, was 5.8. Analysis of some results from the experiment on virulence of *A. tumefaciens* strain LBA4404 in red clover and tobacco explants demonstrated that there were no differences in callus weight and number of shoots between acidic and neutral medium in the absence of AS (data not presented), which further supports the influence of AS on virulence, particularly for red clover, whose values on acidic medium in the presence of AS are significantly higher.

Effect of AS Concentration

Despite the vast literature indicating that AS is a potent activator of the virulence genes in *Agrobacterium*, some detrimental effects on bacterial growth have been reported (e.g., Fortin et al., 1992; 1993). In our

experiments, high concentrations of AS seemed to hinder the growth of *Agrobacterium* in the initial growth phases up to 36 hours after inoculation, but the effect was not observed 72 h after inoculation, when concentrations of AS did not affect growth, as was observed with pH. Dion (1995) corroborates these findings, and states that, in most cases, inhibition of growth due to AS is characterized by an increase in generation time.

Fortin et al. (1992) observed that nopaline-type Ti plasmids exhibited retarded growth in the presence of AS, but octopine plasmids did not. In the present experiment, however, no significant differences were found in the response of the nopaline-strain T37, compared to the octopine strains EHA101, LBA4404, and A281.

The effect of AS on the virulence of *A. tumefaciens* is probably the most commonly reported benefit of AS in transformation experiments. Induction of the *vir* operon can be assessed indirectly, by plant transformation, or directly, by fusibg a *vir* gene to a readily assayable reporter gene, such as *lacZ* (Gartland, 1995). In some cases, very low concentrations of AS (20 µM) have produced a marked effect; for instance, Sheikholeslam and Weeks (1987) showed a 30-fold increase in transformation of *Arabidopsis* when this concentration of AS was used. In the present experiments, AS concentrations of 80 and 320 µM significantly increased the apparent rate of transformation

of red clover and tobacco explants, when *A. tumefaciens* strain LBA4404(pBI121) was used (Table 4-11). In addition, it can be observed that callus weight and number of shoots in tobacco were higher when an intermediate concentration of AS(80 µM) was used; in fact, callus growth and number of shoots were reduced when 320 µM AS were used, possibly indicating some toxic effect of AS on tobacco explants. Similar effects were not observed with red clover explants.

In some examples utilizing *Brassica* species, Holbrook and Miki (1985), Ohlssen and Ericksson (1988), and Charest et al. (1989), observed that the infectivity of this species by octopine strains could be increased by AS. Those researchers postulated that octopine strains, such as LBA4404, may be less sensitive to inducing signals from wounded cells. Furthermore, it was also observed that tobacco cells were able to elicit response, and therefore, effects of AS in this species were less pronounced. A similar trend was observed in our work, since AS caused a three-fold increase in red clover callus weight, as opposed to two-fold increase in tobacco callus weight. This, however, may not hold true for all octopine strains tested (Table 4-13).

Effect of Bacterial Strain

There were differences in the responses of *Agrobacterium* strains to AS concentrations and pH levels.

Strain T37(pMON9793) had the highest OD_{600} at 12, 36 and 72 h of growth. Strains EHA101(pMPSV4-43/20) and EHA101(pMON9793) were different in their apparent ability to induce transformation on red clover explants (Table 4-13). Since the *vir* genes, which are involved in bacterial interactions with AS, are part of the helper plasmid pEHA101, and not part of the binary plasmids, it seems that there was some interaction between the two systems, in such a way that the *vir* genes interacted differently with the two introduced plasmids (pMPSV4-43 and pMON9793). It is also possible that the expression of the *npt*II genes in the two plasmids is not the same, plasmid pMON9793 presenting weaker expression than pMPSV4-43.

An important difference between octopine- and nopaline-type strains lies in the presence of genes *vir*F and *vir*H (=*pin*F) in octopine strains, which are absent from nopaline strains (John and Amaseno, 1988; Garthland, 1995). AS was reported to interact with the *vir*F products, and therefore octopine strains would be more affected by this phenolic compound.

Concluding Remarks

In general, the results in our experiments seem to support the view that acidic conditions, coupled with the addition of AS to the medium, are more conducive to plant transformation by *A. tumefaciens*. Furthermore, these

conditions retarded the growth of *Agrobacterium* and *Escherichia*, although 72 h after inoculation these effects were no longer apparent. Tobacco was more readily transformed by strain LBA4404 than red clover, but the rate of red clover transformation was significantly increased by AS. It is also important to note that medium acidity per se may have negative effects on callus formation, as can be seen by the reduction in callus growth when red clover explants were exposed to acidic YEP medium.

Table 4-1. Bacterial strains of *Agrobacterium tumefaciens* and *Escherichia coli*, with the respective plasmids, used in the experiments testing the effect of acetosyringone and medium pH on the growth of both species and on the virulence of *A. tumefaciens*.

Strain	Chromosomal Background	Ti plasmid		vir region	Binary plasmid	Additional information
		Derivative	Parent			
				A. tumefaciens		
EHA101	C58	pEHA101	pTiBo542	oct† agr LL-sap	none	No T-DNA; derivative of A281(pTiBo542); pEHA101 is a disarmed pTiBo542
					pMON9793	*nptII* from *Tn5*
					pMPSV4-43	*psv-cp* from isolate ER
z707	C58	pSZ707	pTiC58	nop	pMPSV4-43	*psv-cp* from isolate ER *nptI* from *Tn903*; strain C58 used was resistant to nalidixic acid
LBA4404	Ach5	pAL4404	pTiAch5	oct agr	none	No T-DNA; derived from strain LBA4213
					pBI121	pBI121 has *nptII* and *gus* genes
T37	T37	pTiT37	pTiT37	nop agc	pMON9793	Wild-type Ti plasmid
A281	C58	pTiBo542	pTiBo542	oct LL-sap agr lop	pGA482GG	Wild-type Ti plasmid; conjugation of strains A136 and Bo542; A136 is C58 without Ti plasmid.
				E. coli		
HB101	-	-	-	-	pMON9793	*recA hsd*R Str^r
mm294	-	-	-	-	pMON9793	*endA hsd*R

† LL-sap, L,L-succinamopine; lop, leucinopine; agr, agropine; nop, nopaline; agc, agrocinopine; oct, octopine; underlined opine refers to the opine type for that strain.

Table 4-2. Mean squares from the analyses of variance for bacterial growth, estimated by the optical density at 600 nm (OD_{600}) 12 and 36 hours (h) after inoculation, and for final medium pH 36 h after inoculation, for *Agrobacterium tumefaciens* and *Escherichia coli* strains growing on YEP medium at two pH levels (medium pH 5.2 and 7.0) and three concentrations of added components to the medium (50 µM of acetosyringone, 10 mM ethanol, or YEP medium alone). Bacteria were grown at 28 °C in a shaker at 240 rpm.

Source of Variation	df	Bacterial growth		Final medium pH
		12 h	36 h	
Strain (S)	13	0.544**	0.760**	0.222**
Medium pH (M)	1	3.705**	8.123**	115.209**
S X M	13	0.012**	0.027	0.158**
Concentration	2	0.000	0.015	0.002
Error	222	0.001	0.037	0.011

** F test significant at the 1% level.

Table 4-3. Effect of bacterial strain, concentration of added components to the medium, and medium pH on the growth of *Agrobacterium tumefaciens* and *Escherichia coli* strains, estimated by the optical density at 600 nm (OD_{600}), and on change of medium pH. OD_{600} was measured 12 and 36 hours (h) after inoculation, and final medium pH 36 h after inoculation. Bacteria were grown at 28 °C in a shaker at 240 rpm.

	Bacterial growth				Final medium pH	
	12 h		36h			
Treatment	pH 7.0	pH 5.2	pH 7.0	pH 5.2	pH 7.0	pH 5.2
	---------- (OD_{600}) ----------					
Strain†						
T37(pMON9793)	0.87a	0.60a	1.21c	0.82c	6.8b	5.3d††
Z707(pMPSV4-43)	0.83b	0.46b	1.54a	1.12a	6.9b	5.5c
LBA4404	0.53c	0.25c	1.33b	1.00b	6.5d	5.7a
EHA101(pM*/20)	0.38d	0.12de	1.06e	0.74cd	7.1a	5.6ab
EHA101(pMON9793)	0.37d	0.12de	1.10de	0.72cd	7.1a	5.6ab
EHA101	0.36de	0.13d	1.04e	0.72cd	7.1a	5.6ab
EHA101(pM*/1)	0.35def	0.12def	1.04e	0.72cd	7.0a	5.6ab
EHA101(pM*/30)	0.35def	0.11def	1.06e	0.71cd	7.0a	5.6ab
EHA101(pM*/28)	0.34def	0.11def	1.03e	0.70cd	7.1a	5.6ab
EHA101(pM*/11)	0.34def	0.11def	1.05e	0.69cd	7.0a	5.6ab
EHA101(pM*/29)	0.32ef	0.10def	1.07e	0.67d	7.0a	5.6ab
EHA101(pM*/15)	0.31f	0.10def	1.06e	0.68d	7.0a	5.6ab
mm294(pMON9793)	0.32def	0.09ef	1.16cd	0.61d	6.6cd	5.5bc
HB101(pMON9793)	0.21g	0.08f	0.47f	0.28e	6.7c	5.5bc
Concentration of added components						
None (YEP alone)	0.30a		0.90a		6.9a	5.6a
YEP+50 uM AS	0.30a		0.93a		6.9a	5.6a
YEP+10 mM alcohol	0.30a		0.90a		6.9a	5.6a
Medium pH						
Neutral (7.0)	0.42a		1.09a		6.9a	
Acidic (5.2)	0.18b		0.73b		5.6b	

† HB101 and mm294 are strains of *E. coli*; the remaining are *A. tumefaciens*; pM* indicates the plasmid pMPSV4-43.
†† Means with the same letter within columns do not differ at the 5% level (Duncan's multiple range test).

Table 4-4. Mean squares from the analyses of variance for bacterial growth, estimated by the optical density at 600 nm (OD_{600}) 12 h after inoculation, and for final medium pH 36 h after inoculation, for *Agrobacterium tumefaciens* strains A281(pGA482GG), EHA101(pMPSV4-43/20), EHA101(pMSV4-43/30), LBA4404(pBI121), T37(pMON9793), and EHA101(pMON9793), on YEP medium at two pH levels (medium pH 5.2 and 7.0) and eight concentrations of acetosyringone (0, 5, 10, 20, 40, 80, 160, and 320 µM). Bacteria were grown at 28 °C in a shaker at 240 rpm.

Source of variation	df	Bacterial Growth	Final medium pH
Strain (S)	5	1.028**	0.367**
Medium pH (M)	1	40.820**	181.935**
S X M	5	0.108**	0.199*
Concentration (C)	7	0.025**	0.015
Error	173	0.004	0.070

*, ** F test significant at the 5% and 1% levels, respectively.

Table 4-5. Effect of bacterial strain, concentrations of acetosyringone (μM), and medium pH on the growth of *Agrobacterium tumefaciens* strains, estimated by the optical density at 600 nm (OD_{600}), and on change in medium pH. OD_{600} values were recorded 12 h after inoculation, and final medium pH 36 h after inoculation. Bacteria were grown at 28 °C in a shaker at 240 rpm.

Treatment	Bacterial growth		Final medium pH	
	Medium pH 7.0	Medium pH 5.2	Medium pH 7.0	Medium pH 5.2
	---- (OD_{600}) ----			
A. tumefaciens strain				
T37(pMON9793)	1.46a	0.45ab	7.8ab	5.8c†
EHA101(pMON9793)	1.39bc	0.47a	7.9a	6.1a
LBA4404(pBI121)	1.42ab	0.42bc	7.7b	5.8bc
EHA101(pMPSV4-43/30)	1.35c	0.40c	7.9ab	6.0ab
EHA101(pMPSV4-43/20)	1.35c	0.38c	7.9a	6.0ab
A281(pGA482GG)	0.83d	0.13d	7.9a	5.7c
Concentration of AS				
0	1.32a	0.37ab	7.9a	5.9a
5	1.29a	0.42a	7.9a	5.9a
10	1.32a	0.41a	7.8a	5.9a
20	1.32a	0.40a	7.8a	5.9a
40	1.32a	0.39a	7.8a	5.9a
80	1.30a	0.37ab	7.9a	5.9a
160	1.26a	0.34bc	7.9a	5.8a
320	1.25a	0.30c	7.9a	6.0a
Medium pH				
Neutral (7.0)	1.30a		7.9a	
Acidic (5.2)	0.38b		5.9b	

† Means with the same letter within columns do not differ at the 5% level (Duncan's multiple range test).

Table 4-6. Mean squares from the analyses of variance for bacterial growth, estimated by the optical density at 600 nm (OD_{600}) 36 and 72 hours (h) after inoculation, for *Agrobacterium tumefaciens* strains A281(pGA482GG), EHA101(pMPSV4-43/20), EHA101(pMSV4-43/30), LBA4404(pBI121), T37(pMON9793), and EHA101(pMON9793), on YEP medium at two pH levels (5.2 and 7.0) and four concentrations of acetosyringone (0, 20, 80 and 320 µM). Bacteria were grown at 28 °C in a shaker at 240 rpm.

Source of variation	df	Bacterial growth	
		36 h	72 h
Strain (S)	5	0.377**	0.007*
Medium pH (M)	1	0.081**	0.143**
S X M	5	0.007**	0.002
Concentration (C)	3	0.006**	0.002
S X C	15	0.002	0.001
M X C	3	0.001	0.001
Error	15	0.001	0.002

*, ** F test significant at the 5 and 1% levels, respectively.

Table 4-7. Effect of bacterial strain, concentrations of acetosyringone (μM), and medium pH on the growth of *Agrobacterium tumefaciens*, estimated by the optical density at 600 nm (OD_{600}). OD_{600} values were recorded 36 and 72 h after inoculation. Bacteria were grown at 28 °C in a shaker at 240 rpm.

Treatment	36 h		72 h	
	Medium pH 7.0	Medium pH 5.2	Medium pH 7.0	Medium pH 5.2
	-------- (OD_{600}) ---------			
A. *tumefaciens* strain				
T37(pMON9793)	1.49a	1.49a	1.89a	1.93bc†
EHA101(pMON9793)	1.01c	0.98b	1.79b	1.95ab
EHA101(pMPSV4-43/20)	1.07b	0.92bc	1.82ab	1.94ab
A281(pGA482GG)	0.99c	0.90c	1.82ab	1.94ab
EHA101(pMPSV4-43/30)	0.99c	0.88c	1.86ab	1.97a
LBA4404(pBI121)	1.00c	0.86c	1.77b	1.90c
Concentration of AS				
0	1.10a	1.01b	1.79a	1.93a
20	1.10a	1.10a	1.83a	1.95a
80	1.10a	1.01b	1.82a	1.93a
320	1.07a	0.97b	1.85a	1.93a
Medium pH				
Neutral (7.0)	1.09a		1.82b	
Acidic (5.2)	1.00b		1.93a	

† Means with the same letter within columns do not differ at the 5% level (Duncan's multiple range test).

Table 4-8. Mean squares from the analysis of variance for callus weight, in mg, for red clover explants immersed for a few seconds on YEP medium at two pH levels (5.2 and 7.0) and cultivated on B5 callus medium for 12 weeks.

Source of variation	df	Callus weight
Medium pH	1	2080392 **
Error	49	296602

** F test significant at the 1% level.

Table 4-9. Effect of medium pH on callus growth in red clover explants immersed for a few seconds on YEP medium at two pH levels and cultivated on B5 callus medium for 12 weeks.

Treatment	Callus weight
	----- mg -----
Medium pH 7.0	192.5a†
Medium pH 5.2	151.7b

† Means with the same letter within columns do not differ at the 5% level (Duncan's multiple range test).

Table 4-10. Mean squares from the analyses of variance for callus weight, for red clover, and for callus weight and number of shoots, for tobacco cocultivated with *Agrobacterium tumefaciens* strain LBA4404(pBI121) on YEP medium at two pH levels (5.2 and 7.0) and three concentrations of acetosyringone (0, 80 and 320 µM). Calli were cultivated on medium with 50 µg mL^{-1} (red clover) or 100 µg mL^{-1} kanamycin (tobacco), and 300 µg mL^{-1} carbenicillin (both plant species). Callus weight and number of shoots were recorded 35 days after cocultivation.

Source of variation	df	Red clover callus weight	Tobacco	
			Callus weight	Number of shoots
Medium pH (M)	1	1065293*	59306613	38.5
Concentration (C)	2	761224*	153991110**	58.5**
M X C	2	64789	54202370	39.6
Error (clover)	35	207737		
Error (tobacco)	69		34422769	17.6

*, ** F test significant at the 5% and 1% levels, respectively

Table 4-11. Effect of concentrations of acetosyringone (µM) and medium pH on callus weight and number of shoots for red clover and tobacco calli cocultivated with *Agrobacterium tumefaciens* strain LBA4404(pBI121) on YEP medium. Calli were cultivated on medium with 50 µg mL^{-1} (red clover) or 100 µg mL^{-1} kanamycin (tobacco), and 300 µg mL^{-1} carbenicillin (both plant species). Callus weight and number of shoots were recorded 35 days after cocultivation.

Treatment	Red clover callus weight	Tobacco	
		Callus weight	Number of shoots
	mg		
Concentration of AS			
0	26.1b	410.4b	1.5b†
80	70.4a	880.1a	4.4a
320	70.4a	731.5ab	2.9ab
Medium pH			
Neutral (7.0)	31.5b	813.8a	4.0a
Acidic (5.2)	66.6a	600.0a	2.4a

† Means with the same letter within columns do not differ at the 5% level (Duncan's multiple range test).

Table 4-12. Mean squares from the analysis of variance for callus weight, in mg, for red clover cocultivated with *Agrobacterium tumefaciens* strains EHA101(pPSV4-43/30), LBA4404(pBI121), A281(pGA482GG), and EHA101(pMON9793), on YEP medium (pH 5.2) under three concentrations of acetosyringone (0, 80 and 320 µM). Calli were cultivated on B5 medium with 50 µg mL^{-1} kanamycin and 300 µg mL^{-1} carbenicillin. Callus weight was recorded 35 days after cocultivation.

Source of variation	df	Callus weight
Strain (S)	3	1215096**
Concentration (C)	2	268562
S X C	6	304601
Error	112	223153

** F test significant at the 1% level.

Table 4-13. Effect of bacterial strain and concentrations of acetosyringone (µM), on callus weight for red clover explants cocultivated with *A. tumefaciens* grown on YEP medium at pH 5.2. Calli were cultivated on B5 medium with 50 µg mL^{-1} kanamycin and 300 µg mL^{-1} carbenicillin. Callus weight was recorded 35 days after cocultivation.

	Callus weight
	-- mg --
A. tumefaciens strain	
EHA101(pPSV4-43/30)	70.1a†
LBA4404(pBI121)	66.6a
A281(pGA482GG)	63.9a
EHA101(pMON9793)	33.4b
Concentration of AS	
0	46.6a
80	62.0a
320	57.0a

† Means with the same letter within columns do not differ at the 5% level (Duncan's multiple range test).

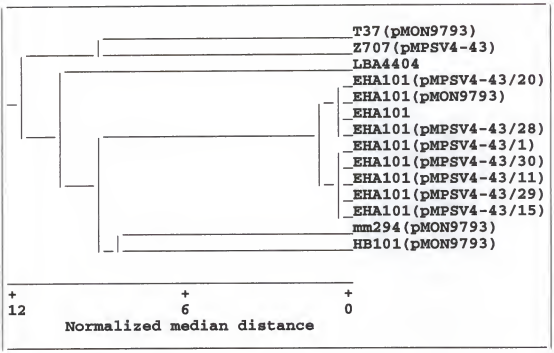

Fig. 4-1. Dendrogram of *Agrobacterium tumefaciens* and *Escherichia coli* strains from a cluster analysis based on bacterial growth 12 and 36 h after inoculation, estimated by the optical density at 600 nm, and for final medium pH 36 h after inoculation. Bacteria were grown on YEP medium on a shaker at 28 °C. Strains mm294 and HB101 are *E. coli*, the remaining are *A. tumefaciens*. Clustering was performed using the complete linkage method, with data standardized to mean 0 and variance 1.

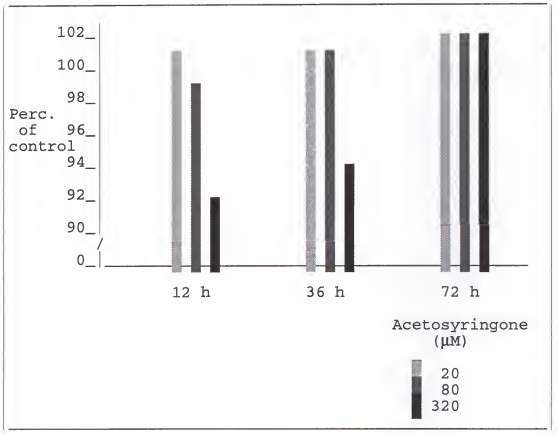

Fig. 4-2. Effect of acetosyringone concentration (AS) on growth of *Agrobacterium tumefaciens*, as a percent of control (Perc. of control; control= 0 AS concentration). Optical density readings at 600 nm taken 12, 36 and 72 hours (h) after inoculation were combined from two experiments. Bacteria were grown at 28 °C in a shaker at 240 rpm.

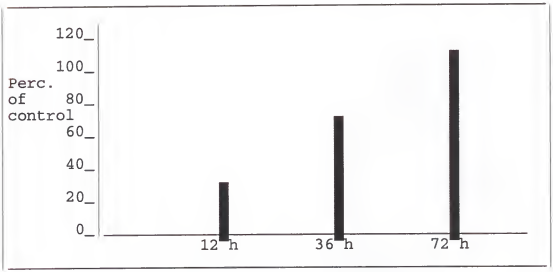

Fig. 4-3. Effect of medium acidity (pH 5.2) on growth of *Agrobacterium tumefaciens*, as a percent of control (Perc. of control; control= growth observed in medium at pH 7.0). Optical density reading at 600 nm were taken 12, 36 and 72 hours (h) after inoculation.

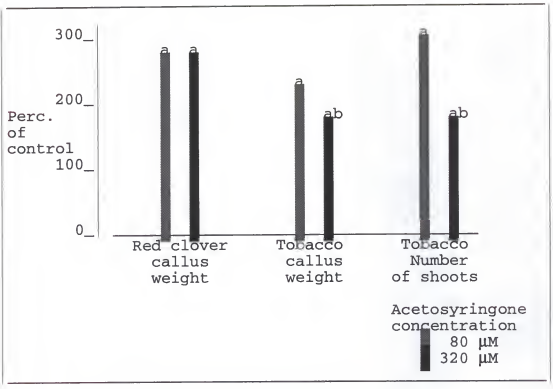

Fig. 4-4. Effect of concentration of acetosyringone (AS) on callus weight and number of shoots for red clover and tobacco, as a percentage of control (Perc. of control; control= callus weight or number of shoots in medium without AS). **Methods**: plant transformation was performed by immersing explants (petiole parts for red clover or leaf discs for tobacco) in *Agrobacterium tumefaciens* strain LBA4404(pBI121 cultures grown on YEP liquid medium with the given concentrations of AS, coculturing for two days in solid medium, then transferring to solid medium containing 300 µg mL^{-1} carbenicillin, to eliminate *A. tumefaciens*, and 50 µg mL^{-1} kanamycin (red clover) or 100 µg mL^{-1} kanamycin (tobacco), to allow only transformed cells to develop. Data were recorded 35 days after cocultivation. Bars with the same letter within each group do not differ at the 5% level (Duncan's multiple range test).

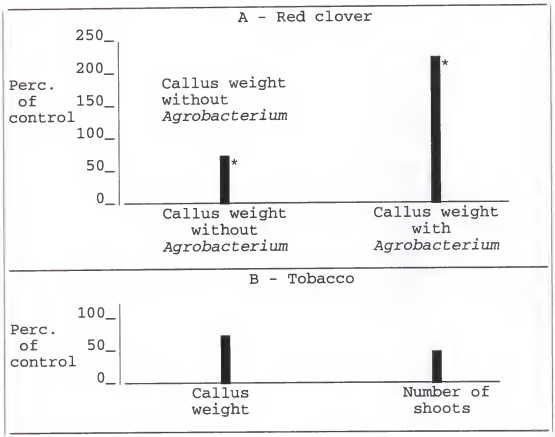

Fig. 4-5. Effect of medium acidity (pH 5.2) on callus weight and number of shoots for red clover and tobacco explants cultivated in B5 medium pH 5.8, after exposed to YEP medium pH 5.2 with or without *A. tumefaciens*, tabulated as a percent of control (Perc. of control; control= callus weight or number of shoots for explants exposed to medium pH 7.0, with or without *A. tumefaciens*). A - Red clover. The diagram on the left shows the effect of medium acidity on callus growth from explants exposed to YEP medium at the two pH levels, in the absence of *Agrobacterium tumefaciens*, and cultivated in B5 medium without antibiotics; the diagram on the right shows the effect of medium acidity on callus growth from explants exposed to *A. tumefaciens* which was grown on YEP medium at the two pH levels and several concentrations of acetosyringone (see Materials and Methods). Explants were cocultivated with *A. tumefaciens* for 48 hours, then transferred to medium with 300 μg mL^{-1} carbenicillin, to eliminate the bacteria, and 50 μg mL^{-1} kanamycin, to select for transformed cells. B - Tobacco. Explants were exposed to *A. tumefaciens* as above, and transferred to selective medium containing 100 μg mL^{-1} kanamycin. Left: effect of medium pH on callus weight; right: effect on number of shoots. The symbol * indicates that the treatment was statistically different from its control at the 5% level (Duncan's multiple range test).

CHAPTER 5
AGROBACTERIUM-MEDIATED TRANSFORMATION OF RED CLOVER AND TOBACCO WITH THE PEANUT STUNT VIRUS COAT PROTEIN GENE

Introduction

The bacterial genus *Agrobacterium* is well-known for its unique capacity to transfer DNA into plant cells. The transferred DNA (T-DNA) is part of a large plasmid, the tumor-inducing plasmid (pTi). The T-DNA is integrated into the plant DNA, and subsequent expression of its genes leads to the formation of tumors. Genetically-engineered strains of *Agrobacterium* are available in which the virulent, tumor-inducing genes, are eliminated, allowing their use as vectors for desirable genes (Binns, 1990; Gasser and Fraley, 1989). Most available vectors contain scorable and antibiotic-resistance genes, allowing assay of plants in which they have been incorporated.

In plants, cross protection is a method used to confer resistance to viruses. This is achieved by inoculating the crops with a mild strain of the virus for which protection is intended. Some of the drawbacks of cross protection include the necessity for large-scale field infections with the mild strain, and the reduction in yield which is

generally associated with the infection (Gonsalves and Fulton, 1977).

Progress in molecular genetics and plant transformation techniques has made genetically-engineered virus resistance using cloned viral genes possible. This system has been demonstrated in several plant and animal systems. Coat protein-mediated resistance is the most common method of genetically-engineered virus resistance. Only genes responsible for the coat protein (CP) are included in the construct. Other approaches include the use of satellite sequences, antisense RNAs, and the whole genome of a mild virus. Yet other strategies, such as interference with viral replication by sense RNAs or by defective interfering particles, or the use of transgenic plants that constitutively express pathogenesis-related proteins, have been suggested (Grumet, 1990).

Red clover (*Trifolium pratense* L), an important legume that provides good quality forage, is susceptible to infection by peanut stunt virus (PSV), which causes systemic mottle in the plants. Likewise, tobacco (*Nicotiana tabacum* L.) is susceptible to this same virus.

The present project explores the use of coat protein-mediated resistance. *A. tumefaciens* harboring a plasmid with the coat protein gene from PSV was used to transform tobacco and red clover explants, and the effectiveness of T-DNA transfer was assessed by several methods.

Materials and Methods

Bacterial Strains and Plasmids

Agrobacterium tumefaciens strain Z707, transformed with the plasmid pMPSV4-43, a derivative of pMON530 containing the gene coding for the PSV-CP, and *A. tumefaciens* strain EHA101 transformed with the plasmids pMPSV4-43 and pMON9793, which harbor both the *nptII* and *gus* genes, were used in this study. *A. tumefaciens* Z707(pMPSV4-43) was obtained from Dr. S. Ghabrial, University of Kentucky, who also sent purified plasmid pMPSV4-43, and antiserum against PSV-CP. *A. tumefaciens* EHA101, both non-transformed and transformed with pMON9793, were obtained from glycerol stocks maintained at our laboratory.

The plasmid pMON530, a binary cassette vector (Rogers et al.,1987) contains 12034 base-pairs (bp) and a synthetic multilinker. A 3.8 kilo-base (kb) *Hind*III-*Sma*I segment of the mini RK2 plasmid, pTJS75, which contains the RK2 origin of replication, *ori*V, and the origin of transfer, *ori*T, for conjugation into *A. tumefaciens* using the triparental mating procedure, is also present. This permits independent replication in *Escherichia coli*. This fragment is followed by a 1.6 kb segment carrying the pBR322 origin of replication, for ease of making large amounts of the vector in *E. coli*, and a 2.4 kb segment of the nopaline-type pTiT37 plasmid, that carries the right border of the nopaline T-

DNA, and an intact nopaline synthase (*nos*) gene. This fragment allows facile scoring of transformants, and also permits easy scoring of progeny in inheritance studies. Bacterial antibiotic resistance is given by a 2.2 kb segment, originating from *Tn*7, carrying the spectinomycin and streptomycin resistance genes. Resistance of plants to kanamycin is provided by a 1.6 kb-long segment, encoding a chimeric *nos-npt*II-*nos* gene. Transcription is directed by the CaMV 35S promoter segment, from the cauliflower mosaic virus. In addition, there is a *nos*-3' nontranslated sequence. The synthetic multilinker, located between these two fragments, contains 35 bp and sites for the following restriction enzymes: *Bgl*II, *Cla*I, *Sma*I, *Kpn*I, *Sal*I, and *Eco*RI. The gene coding for the PSV coat protein (*psv-cp*) was cloned into the synthetic multilinker as a fragment digested by *Cla*I and *Kpn*I. The nucleotide sequence of the cDNA of clone pMPSV4-43, used in the present project and originating from isolate PSV-V, was determined by Naidu et al. (1991a), and is part of the EMBL, GenBank and DDBJ Nucleotide Sequence Databases, where it appears as accession number X56544. The cDNA contains 986 bp. The open reading frame (ORF) is 675 bp in length, and codes for a capsid protein of 224 amino acid residues, with molecular weight of 24.9 kDa.

Mobilization of the Binary Vector pMPSV4-43 into *Escherichia coli*

The plasmid pMPSV4-43 was initially introduced into three strains of *E. coli*: Subcloning Efficiency DH5-α Competent Cells, Max-Efficiency DH5-α Competent Cells (both from Gibco BRL Laboratory), and LB1-Blue cells made competent by the calcium chloride method, as described below.

LB1-Blue cells were grown overnight in YEP medium (in mg mL^{-1}: peptone, 10, yeast extract, 10, sodium chloride, 5), with phosphate buffer, pH 7.0. The next morning the cultures were centrifuged for 5 min at 7,000 rpm, the supernatant was discarded, and the pellet was resuspended in cold, sterile 100 mM calcium chloride for 20 min on ice. The cells were again centrifuged as before, resuspended in minimal volume of 100 mM calcium chloride with 15% glycerol, and frozen at -80 °C. The LB1-Blue competent cells were tested using SK(-) DNA. One µL of a 1 ng $µL^{-1}$ SK(-) DNA solution was mixed with 100 µL competent cells, kept on ice for 1 hour (h), then transferred to 42 °C for 1 min. YEP medium (300 µL) was added, and the cells plated on YEP plates with 75 µg mL^{-1} ampicillin, followed by incubation at 37 °C.

The transformation with the plasmid pMPSV4-43 followed the procedures recommended by the BRL Technical Services Department. Competent cells were mixed with aliquots of the

purified pMPSV4-43 plasmid and incubated on ice for 30 min. A 20-sec heat shock (37 °C) followed. Cells were then mixed with S.O.C. medium (2% triptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose) and kept overnight at room temperature. Selection of transformed *E. coli* was done by plating aliquots on Petri dishes containing solid YEP medium with 30 μg mL^{-1} streptomycin sulfate (Sigma Chemical Co.). Glycerol stocks were maintained at -20 °C and -70 °C. Plasmid DNA was subsequently isolated from *E. coli* following a modified mini alkaline-lysis/PEG (polyethylene glycol) precipitation procedure. Cells were lysed with 0.2 N sodium hydroxide/1% lauryl sulfate, sodium salt (SDS), cellular debris removed by centrifugation, and the supernatant extracted with chloroform. DNA from the aqueous phase was precipitated with isopropanol followed by centrifugation. Plasmid DNA was purified by redissolving the pellet in water, followed by 4 M NaCl and 13% PEG. The pellet was resuspended and maintained in $T_{10}E_1$ (10 mM Tris, 1 mM EDTA, pH 8.8). Restriction-digestion of the isolated plasmid DNA with EcoR1 was performed to assess the integrity of the plasmids.

Mobilization of the Binary Vector pMPSV4-43 into *A. tumefaciens* Strain EHA101

Cells of *A. tumefaciens* EHA101 at the log phase of growth were made competent by the calcium chloride method.

The cells were chilled on ice and centrifuged at 5,000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet resuspended in minimal volume of cold 20 mM calcium chloride. Direct transformation was performed by adding approximately 1 µg of plasmid DNA to 1 mL competent cells, followed by freezing in liquid nitrogen and thawing at 37 °C for 5 min. One mL of YEP was added to the cells, which were allowed to grow for 2 h at 28 °C. The cells were pelleted for 30 sec in a microcentrifuge and resuspended in 100 µL YEP medium. Cells were selected on solid YEP medium containing 100 µg mL^{-1} spectinomycin, 50 µg mL^{-1} kanamycin and 50 µg mL^{-1} streptomycin. The colonies were further tested for ability to grow in YEP liquid medium containing streptomycin, kanamycin and spectinomycin at the same concentrations, against growth of non-transformed *A. tumefaciens* EHA101.

PCR was used to assess the presence of the plasmid in the putative transformed cells. A single bacterial colony was boiled in 100 µL of water for 1 min, and mixed with the following components: 10x PCR buffer (500 mM potassium chloride, 100 mM Tris-HCl, pH 8.3, 15 mM magnesium chloride, and 0.1% (w/v) gelatin), 8 µL 2.5 mM dNTP's, 1 ug forward primer, 1 ug reverse primer, and 2.5 units (U) Taq polymerase (Perkin Elmer/Cetus). The PCR conditions were: DNA denaturing at 94 °C for 1 min and 30 sec, primers annealing at 60 °C for 1 min and 30 sec, and elongation at

72 °C for 2 min and 30 sec. The cycle was repeated 30 times. Primers for the *psv-cp* and *npt*II genes, which are described later, were used.

Plant Materials, Culture Conditions, and Recovery of Putative Transgenic Plants

Tobacco cultivar Xanthi, and red clover germplasm NEWRC (Smith and Quesenberry, 1995), were used in the transformation experiments. This red clover exhibits a high frequency of *in vitro* regeneration (Quesenberry and Smith, 1990), and has known potential for transformation with *A. tumefaciens* strain EHA101 (Quesenberry et al., 1992). Plants were grown from seeds under sterile conditions. The seeds were germinated in Petri dishes and transferred to Magenta boxes containing B5 medium (Gamborg et al., 1968) from Sigma (Sigma Chemical Co, St. Louis, MO; in $\mu g\ mL^{-1}$: ammonium sulfate, 134.0; boric acid, 3.0; calcium chloride anhydrous, 113.24; cobalt chloride, 0.025; cupric sulfate, 0.025; Sodium-EDTA, 37.25; ferrous sulfate, 27.85; magnesium sulfate, 122.09; manganese sulfate, 10.0; molybdic acid (sodium salt), 0.25; potassium iodide, 0.75; potassium nitrate, 2500.0; sodium phosphate monobasic, 130.5; zinc sulfate, 2.0; myo-inositol, 100.0; nicotinic acid (free acid), 1.0; pyridoxine-HCl, 1.0; thiamine-HCl, 10.0; pH adjusted to 5.8; energy source, 20 g l^{-1} sucrose).

A. tumefaciens cultures were initiated from the glycerol stocks in either Luria Bertani medium (LB;

transformation involving *A. tumefaciens* Z707) or YEP medium (transformation involving *A. tumefaciens* EHA101). Upon reaching the logarithmic phase of growth, the cell density was adjusted to approximately $5x10^8$ colony-forming units (CFU) mL^{-1} by centrifuging the culture and resuspending the cells in the appropriate amount of medium without antibiotics.

Red clover transformation. Petiole parts (from 4 to 8 mm in length) from red clover were briefly immersed in *A. tumefaciens* liquid cultures, and placed in Petri dishes containing B5 callus medium, where they remained for 48 h (cocultivation period). After cocultivation, the petiole explants were transferred to B5 callus medium (B5 basal salts and vitamins plus: α-naphtaleneacetic acid (NAA), 2.0 μg mL^{-1}; 2,4-dichlorophenoxiacetic acid (2,4-D), 2.25 μg mL^{-1}; kinetin, 2.125 μg mL^{-1}), supplemented with 300 μg mL^{-1} carbenicillin, to eliminate *A. tumefaciens*, and 50 μg mL^{-1} kanamycin, to select for transformants, where they remained for a period of four to six weeks. Calli were transferred to B5 embryo induction medium (B5 basal salts and vitamins plus NAA, 2.0 μg mL^{-1}; adenine, 2.0 μg mL^{-1}), with the same concentration of antibiotics as described above. They remained in this medium for a period sufficient to develop somatic embryos, usually six to eight weeks; if necessary, they were transfered to new medium after 4 to 6 weeks. Plantlets were transferred to B5 rooting medium (B5 basal

salts and vitamins, plus 0.2 μg mL^{-1} mg NAA). All the above phases were conducted in a Percival growth chamber (Percival, Boone, Iowa) at 28 °C and 40 μmol m^{-2} s^{-1} of light from fluorescent lamps.

As somatic embryos developed into whole plants, they were transferred to plastic Magenta™ culture vessels (Magenta Co., Chicago, IL) containing B5 medium without hormones. Eventually, whole plants were transferred to peat balls, where they remained for approximately two weeks with high humidity (adjustment period) prior to being moved to a greenhouse. These phases were conducted at 28 °C and 150 μmol m^{-2} s^{-1} light from fluorescent lamps, on a laboratory bench. A total of 1,900 petiole parts associated with 70 genotypes were subjected to transformation with *A. tumefaciens* strain Z707(pMPSV4-43), during several periods in 1993. Plants regenerated from these experiments were evaluated for transformation using ELISA and PCR.

After transferring the pMPSV4-43 plasmid into *A. tumefaciens* strain EHA101, a similar procedure was followed for red clover transformations in 1995. Approximately 300 petiole parts were subjected to transformation, using several isolates of this strain.

Tobacco transformation. Leaf discs of tobacco (6 mm diameter) were used as the source of explants. *Agrobacterium* cocultivation followed the same protocol described for red clover. After cocultivation, the leaf discs were transferred

to MS medium (Murashige and Skoog, 1962) from Sigma (Sigma Chemical Co, St. Louis, MO; in μg mL^{-1}: ammonium nitrate, 1650; boric acid, 6.2; calcium chloride anhydrous, 332.2; cobalt chloride, 0.025; cupric sulfate, 0.025; Sodium-EDTA, 37.25; ferrous sulfate, 27.8; magnesium sulfate, 180.7; manganese sulfate, 16.9; molybdic acid (sodium salt), 0.25; potassium iodide, 0.83; potassium nitrate, 1900.0; potassium phosphate monobasic, 170.0; zinc sulfate, 8.6; glycine (free base), 2.0; myo-inositol, 100.0; nicotinic acid (free acid), 0.5; pyridoxine-HCl, 0.5; thiamine-HCl, 0.1; pH adjusted to 5.7; energy source, 30 g/l sucrose), supplemented with 2 μg mL^{-1} IAA and 2 μg mL^{-1} kinetin plus 300 μg mL^{-1} carbenicillin and 100 μg mL^{-1} kanamycin. The explants were kept at 28 °C in the dark until plantlets started to form, when they were transferred to MS medium without hormones. Plant development was conducted in light as described above. *A. tumefaciens* EHA101 harboring either pMON9793 or pMPSV4-43 was used in the transformation experiments. Approximately 300 leaf discs were exposed to *A. tumefaciens* harboring pMPSV4-43, and 50 leaf discs to *A. tumefaciens* harboring pMON9793, in 1995.

Assay of Putative Transgenic Plants for the Presence and Expression of the *psv-cp* and *nptII* Genes

The presence of the inserted genes was assayed by enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR).

Plate-trapped antigen indirect enzyme-linked immunosorbent assay (PTA-I-ELISA) for detection of PSV-CP

The standard protocol used in most ELISA assays was as follows. One hundred µL of antigen preparation (approximately 1 g leaf tissue processed through a mechanical leaf press irrigated with 2 mL of coating buffer: 12 mM sodium bicarbonate, 35 mM sodium carbonate, pH 9.6) was added to each well (Flat-bottomed well, High-binding ELISA plates; Corning Glass Works, New York) and incubated for 1 h at 37 °C. The plates were rinsed with four 5-min washes in PBS (20 mM sodium phosphate-potassium phosphate buffer, containing 3 mM potassium chloride and 150 mM sodium chloride, pH 7.4) plus 0.05% Tween-20 (PBST). This was followed by blocking with 0.5% bovine serum albumin (BSA) in PBS for 1 h at 37 °C., and washing as before. One hundred µL of antiserum produced against PSV-CP in rabbit, diluted 1:5,000 in PBS, was added to each well, and incubated for 1 h at 37 °C and washed as before. After washing, 100 µL of goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma Chemical), diluted 1:20,000 in PBS was added. The incubation period was as before, followed by washing. One hundred µL of substrate (paranitrophenyl disodium phosphate, 1 mg mL^{-1}, Sigma Chemical) in substrate buffer (9.7% diethanolamine, pH 9.7, Fisher Scientific) was added and incubated at room temperature in the dark. Absorbance readings at 405 nm (A_{405}) were taken on a Biotek automated microplate reader, model EL

309 (Bio-Tek Instruments, Winooski, VT) at intervals. Controls were constituted from healthy plants and plants inoculated with PSV. Each treatment consisted of three wells in a single ELISA plate. In some cases, the experiments were replicated four times in different plates, and the data subjected to the analysis of variance.

An experiment was also set up to examine the effects of extraction buffer and dilution of sap from putatively transformed plants and controls in the ELISA results. Two extraction buffers [PBS, pH 7.4, and sodium carbonate/bicarbonate buffer (=coating buffer), pH 9.6, as described above] and five plant sap dilutions (1:3, 1:6, 1:12, 1:25, and 1:50) were tested. In addition, 200 µM sodium diethylcarbamate (NaDIECA) or 2.0% polyvinylpyrrolidone, 40,000 MW (PVP) were tested in combination with coating buffer. Each treatment consisted of two adjacent wells. Data from both experiments were subjected to analysis of variance, and the means compared through Duncan's multiple range test.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for detection of NPTII

This assay was performed using the NPTII ELISA KIT (5 Prime → 3 Prime, Inc., Boulder, CO), following instructions of the manufacturer. A similar procedure is described by Baszczynsky (1990). Rabbit polyclonal antibody specific to the NPTII protein encoded by *Tn*5 was coated onto polystyrene

microwells. The microwells were then blocked as before to prevent non-specific adsorption. Extracts from putative transformants and from healthy controls, and pure NPTII protein as standard, were added to the wells. Unbound material was removed by washing the wells. Biotinylated secondary antibody to NPTII was added, which would bind to the immobilized primary antibody/NPTII complex. After washing, bound biotinylated antibody was quantitated colorimetrically by incubation with streptavidin conjugated alkaline phosphatase and substrate. The color development was expected to be proportional to the concentration of NPTII protein present in each well.

DNA isolation

DNA was isolated from tobacco and red clover leaves following the protocol described by Dellaporta et al. (1983) with modifications. One gram of leaf tissue was ground in liquid nitrogen. Extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM sodium chloride, 10 mM mercaptoethanol, pH 8.0) was added to the ground leaves while still frozen. When the mixture thawed, SDS (20%) was added, the material transferred to 50-mL centrifuge tubes, and incubated at 65 °C for 10 min, after which 5 M potassium acetate was added, to remove proteins and polysaccharides. The tubes were centrifuged at 25,000xg for 20 min to remove cell debris, and the supernatant poured through Miracloth (Calbiochem

Co., La Jolla, CA) into clean tubes containing cold isopropanol. These tubes were incubated at -20 °C for 30 min to precipitate the DNA, which was then pelleted at 20,000xg for 15 min. The DNA pellets were resuspended in a small volume of 50 mM Tris, 10 mM EDTA, pH 8.0, in Eppendorf tubes, which were centrifuged in a microcentrifuge for 10 min to remove insoluble debris. The supernatant containing DNA was mixed with 7.5 M ammonium acetate and absolute ethyl alcohol, to be finally pelleted in a microcentrifuge. The purified DNA was washed with 70% ethanol, vacuum dried, and resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0.

Assessment of DNA quantity and quality was done using a Perkin-Elmer Lambda-1 UV/VIS spectrophotometer. Optical density readings were taken at 260 nm and 280 nm (OD_{260} and OD_{280}, respectively). OD_{260} estimated the concentration of nucleic acid in the sample, and the ratio OD_{260}/OD_{280} provided an estimate of the purity of the DNA (Sambrook et al., 1989.). Working solutions containing 50 ng μL^{-1} DNA were prepared and kept frozen until used.

DNA amplification by polymerase chain reaction (PCR)

Genomic DNA was amplified using the polymerase chain reaction (PCR). Primers were designed to amplify a 321 base-pair (bp) fragment of the *npt*II gene, and a 878 bp fragment of the *psv-cp* gene. The *npt*II primers were: GAM15 (5' TCA CTG AAG CGG GAA GGG ACT 3') and GAM16 (5' CAT CGC CAT GGG

TCA CGA 3') complementary to nucleotides 1801-1822, and 2101-2122, respectively. The primers for the *psv-cp* gene were: SG3700 (5' dGGC ACC TAA AGG TAC TGG TAA CG 3'), complementary to nucleotides 48-71, and SG3701 (5' dCAG CTC TTC ACA ATC ACC AGG AG 3'), complementary to nucleotides 904-926. The following PCR conditions were used: DNA denaturation at 94 °C for 1 min, primers annealing at 60 °C for 1 min, and chain elongation at 72 °C for 3 min, for 40 cycles, followed by final chain elongation at 72 °C for 5 min. Genomic DNA was used at rates of 50 ng or 300 ng per 50 µL reaction. The other reaction components were (per 50 µL reaction): 10X buffer, 5 µL; 2.5 mM magnesium chloride, 3 µL; 2.5 mM dNTP's, 3 µL; primers, 0.5 µL each; Taq polymerase (Promega; 5 units μL^{-1}), 0.25 µL; and water to complete the volume. The equipment used was an Ericomp TwinBlock System, EasyCycler Series (Ericomp Inc., San Diego, CA), with water circulation provided by an IsoTemp Refrigerated Circulator unit (Fisher Scientific). Amplification products were electrophoresed on 1.2% (*psv-cp*) or 2.0% agarose gel (*npt*II) for 2 hours.

Results

Mobilization of the Binary Vector pMPSV4-43 into *A. tumefaciens* Strain EHA101, and *E. coli*

The plasmid pMPSV4-43 was successfully transferred into *A. tumefaciens*. Thirty-three colonies were harvested which

grew on selective YEP solid medium containing 30 μg mL^{-1} streptomycin. The colonies were further tested for growth in YEP liquid medium containing streptomycin, kanamycin and spectinomycin (in μg mL^{-1}, respectively: 50, 50 and 100), and compared against the growth of non-transformed *A. tumefaciens* strain EHA101. Table 5-1 presents the OD_{600} at 12 and 24 hours for the recovered colonies. Seven colonies were selected for use in transformation experiments [colonies EHA101(pMPSV4-43) 1, 11, 15, 28, 29, and 30]. *A. tumefaciens* transformation was confirmed through PCR, whose results (Fig. 5-1) show an amplified fragment close to the ca. 1,000 bp ladder fragment, matching the expected size of 878 bp.

Transformation of *E. coli* with the same plasmid was not achieved, however, despite seven attempts using three different, high-quality competent strains. Cell colonies were recovered from the solid medium with the appropriate amounts of antibiotics, but PCR amplification of the *psv-cp* and *npt*II genes always failed to reveal the presence of the pMPSV4-43 plasmid in the recovered cells.

Assay of Putative Transgenic Plants for the Presence and Expression of the *psv-cp* and *npt*II Genes

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for detection of NPTII

One ELISA assay was carried out to investigate the presence of putative transgenic red clover plants from the transformations made in 1993, with *A. tumefaciens*

Z707(pPSV4-43). Table 5-2 presents some representative results from this assay. Control, untransformed plants, presented A_{405} values that overlapped those of the putative transgenic plants.

Plate-trapped antigen indirect enzyme-linked immunosorbent assay (PTA-I-ELISA) for detection of PSV-CP

ELISA was carried out on putative transgenic red clover originated from the transformations made in 1993, involving *A. tumefaciens* Z707(pMPSV4-43), and on putative transgenic tobacco from the transformations made in 1995, involving *A. tumefaciens* EHA101(pMPSV4-43) and EHA101(pMON9793). Red clover from the 1995 transformations involving *A. tumefaciens* strain EHA101(pMPSV4-43) was still in the somatic embryo-phase of tissue culture at the time of this report, and therefore could not be evaluated.

ELISA tests were done on ca. 200 putative red clover transgenic plants from the transformation involving *A. tumefaciens* strain Z707(pMPSV4-43) on several occasions in 1994 and 1995 (Tables 5-2 and 5-3). Although high A_{405} values were observed for both the test plants and the positive controls, negative red clover controls also yielded high A_{405} values due to strong background. Negative controls in most assays originated from greenhouse-growing plants, and the possibility of infection with PSV can not be ruled out.

Several ELISA assays were also carried out with tobacco plants on various occasions, and the results were somewhat

variable, meaning that the same plant would show weak or no reaction in some assays, and strong reaction in others. However, a number of plants showed more consistent results, and were selected for further studies. The results from the analysis of variance on one such assay, involving eleven plants of tobacco and three controls, and also some red clover plants, are presented in Table 5-4. There were significant differences between plants and blocks. Table 5-5 shows the A_{405} values for the plants tested, including one positive control (*Vigna sinensis* inoculated with PSV), and four negative controls (two tobacco, one red clover, and one cowpea). The absorbance values range from A_{405}= 2.585 for the positive control to A_{405}= 0.083 for tobacco accession TMON9793-06-6. The mean value for the tobacco negative controls was A_{405}= 0.178, and for the red clover control it was A_{405}=0.199.

The most common method used to set ELISA threshold values is an A_{405} twice the average of the negative controls (Sutula et al., 1986). If such a value is used with these data, values above A_{405}= 0.356 for tobacco, and A_{405}= 0.398 for red clover, would be considered positive. Several the tobacco plants that had been transformed with EHA101(pMPSV4-43) were positive for the presence of the engineered viral coat protein product. Tobacco TMON9793-06-6, which was transformed with a vector that did not carry the gene coding

for PSV-CP, was negative, as expected. All three red clover plants tested were positive.

The second ELISA experiment tested the effect of extraction buffer and anti-oxidants on absorbance values. Table 5-6 presents the mean squares from the analysis of variance, indicating significant differences for extraction buffer, plants, sap dilution, as well as for the interactions of extraction buffer with the other two variables. Table 5-7 presents the A_{405} mean values for all buffers. Sodium carbonate/bicarbonate buffer (=coating buffer) presented higher A_{405} for any given combination plant-sap dilution. The addition of anti-oxidants to coating buffer, or the use of PBS extraction buffer, reduced the A_{405} in all instances, except for the two negative controls. Sap dilution caused a linear reduction in A_{405}.

DNA isolation

DNA was isolated from 14 tobacco plants that had been recovered from kanamycin-containing medium, from the transformation conducted in 1995 with *A. tumefaciens* EHA101(pMPSV4-43), and from 12 red clover plants also recovered from kanamycin-containing medium, from the transformation conducted in 1993 with *A. tumefaciens* Z707(pMPSV4-43). Control plants, non-transformed, were also subjected to DNA isolation. The spectrophotometric determination of the amount of DNA present revealed

variation (data not presented). DNA quality was good in most cases, since the ratio OD_{260}/OD_{280} was frequently 1.8 and above.

DNA amplification

Several attempts to amplify the target DNA sequences from red clover and tobacco were carried out, with varying results. DNA from tobacco plants amplified both the *psv-cp* and the *npt*II fragments, some sources being more consistent than others in the various PCR reactions run. These would include accession TPSV20-11-2, which also presented the highest A_{405} in the ELISA assay (Table 5-5). Some clover plants also amplified the expected bands. Fig. 5-2 and 5-3 present some results from PCR for the *npt*II and *psv-cp* genes, respectively. It is interesting to note that larger amounts of DNA (300 ng/50 µL reaction) were more conducive to amplification by Taq polymerase, since the DNA quantity in the PCR reactions that used this amount was considerably higher than that produced in reactions that used 50 ng DNA (data not presented).

Discussion

Transformation of red clover explants with *A. tumefaciens* strain EHA101 harboring the binary plasmid pMON9793, which codes for resistance to kanamycin and related antibiotics through the expression of the *npt*II

gene, has been demonstrated (Quesenberry et al., 1992). These authors, however, stressed that *A. tumefaciens* strains have been shown to vary in transformation effectiveness among plant species and among genotypes within a species. In that same experiment, it was demonstrated that *A. tumefaciens* strain T37(pMON9793) was inferior to EHA101 harboring the same plasmid, and T37SE::pMON9749 was not effective at all in transforming red clover, clearly indicating differences among bacterial strains and plasmids in their ability to transform plants.

Other examples can be found in the literature reporting wide variation in infectivity for different strains or isolates of *A. tumefaciens*, and also in the reaction of plant species or cultivars to a given strain. Wondragen et al. (1991) found that only a few chrysantemum genotype-*A. tumefaciens* strains combinations resulted in significant transformation. Similar results were reported by Barfield and Pua (1991), Jacq et al. (1993) and Vanhala et al. (1995). Kathan and Jacobsen (1990) found that the *Agrobacterium* chromosomal background, as well as the type of vector construction (binary versus cointegrating), did not influence transformation efficiency in pea, but the *vir*-region had marked effect; virulence could be increased by using *vir*-region fragments from more virulent strains. The authors also found that two out of four strains did not induce tumor formation in this plant species.

Molecular studies also revealed variation in allelic forms of the VirA protein, which could account for differences in rates of transformation (Rainieri et al., 1993). Furthermore, molecular characterization of the VirF protein also revealed differences between strains, nopaline strains lacking a functional form of this protein due to mutation or absence of the gene (Melchers et al., 1990); differences between octapine- and nopaline-type strains are already well established in the literature (Chilton, 1993).

Escapes are also not uncommom in plant transformation experiments. McHughen and Jordan (1989) stated that the problem of escapes is well known to those involved in research with transgenic plants, which could be due to instability of the T-DNA, genomic rearrangements, or non-rigorous selection or identification assays. Some plants may also be chimeric. For example, Parrot et al.(1989) cocultivated 9800 cotyledons of soybean with *Agrobacterium*, and a total of 18 plants were recovered, but only three were transformed; all three were shown to be chimeric, since no progeny of the three plants were transformed. Atkinson and Gardner (1991) found that only ca. 10% of pepino plants recovered from medium containing kanamycin were transgenic.

In citrus, regeneration in the presence of kanamycin may not be a reliable indicator of transformation (Moore et al., 1992). The authors suggested that a small number of surviving bacteria may inactivate the kanamycin in the

medium. The number of regenerated citrus plants that proved positive for GUS in this system ranged from 1.5% to 40% (Gutierrez, 1995; Moore et al., 1989, 1992). Pena et al. (1995), however, stressed that kanamycin selection did enrich for transgenic citrus plants; when no kanamycin was used in the medium, only non-transformed plants regenerated, whereas with kanamycin, 8% were transgenic. This also demonstrated that transgenic plants may have a disadvantage in tissue culture, which reinforces the need for an effective screening method. The relative inefficiency of the selective agent in transformation experiments in many other plant species is well documented in the literature, but, in some cases, most of the regenerated plants are transgenic. For example, Uematsu et al. (1991) obtained a success rate close to 90% in kiwi transformation experiments. In oats, Torbert et al. (1995) were able to greatly improve the percentage of recovered plants that were transgenic for the genes of interest by replacing inefficient kanamycin with paromomycin.

In order to have more than one construct to transform red clover and tobacco, we embarked on a program to transform *A. tumefaciens* strain EHA101, which had been proved effective in transforming red clover, with the plasmid pMPSV4-43, carrying the *psv-cp* gene. This plasmid was successfully introduced into *A. tumefaciens* strain EHA101, although transformation of *E. coli* with the same

plasmid was not achieved, despite several attempts using three different, high-quality competent cell lines. The second step was to transform tobacco and red clover with the new strain of *A. tumefaciens* carrying the desired genes. As a control, *A. tumefaciens* strain EHA101(pMON9793) was used.

We have successfully demonstrated the transformation of tobacco plants with both constructs. Plants that regenerated from the transformation with EHA101(pMON9793) were tested through GUS assays, and a number were shown to be positive. PCR on a GUS-positive tobacco plant, using primers for the *npt*II gene, also showed amplification of the expected band. Tests with plants transformed with EHA101(pMPSV4-43) included ELISA and PCR, and both showed that a number of recovered tobacco plants were positive for both tests. Since red clover requires longer periods in tissue culture before plants are recovered, plants large enough to be tested by ELISA or PCR have not yet been obtained from the transformations involving EHA101(pMPSV4-43) made in 1995. Red clover plants recovered from the transformations involving Z707(pMPSV4-43), on the other hand, showed positive results for both ELISA and PCR.

Several PCR tests were done, and a few plants always produced positive results. Variability was found when different tests were compared. In optimizing the amount of DNA in each 100 µL reaction, 8 µL of a 50 ng μL^{-1} stock gave the best amplification. Hamill et al. (1991) also emphasized

the importance of annealing temperature; for tobacco, they found this to be the most important factor, and amounts of DNA ranging from a few ng to 2 µg per 50 µL reaction produced the same results. However, for mint species, an inhibitor of the PCR process was co-extracted with the DNA which prevented amplification if more than 10 ng of DNA was present in the reaction.

Over the several ELISA tests conducted, variability in results was observed. Some plants showed a weaker reaction in one ELISA test, and a stronger reaction in another ELISA. Two hypotheses may account for this variability. The first is that the regenerated plants are chimeric, and only certain sectors are transformed; and the second one is that the expression of the introduced genes is different, either spatially or temporally. These are not uncommon phenomena in the literature. For example, Fitch et al. (1992) reported differential expression of the ß-glucuronidase gene in papaya plant parts, and even in different parts in the same leaf. Gene silencing was also was observed by Hoffmann et al. (1988) in germinating pollen grains of transformed tobacco. Silencing of genes in transgenic plants is not uncommon, and has been reported for several plant species. The main features were reviewed by Meins and Kunz (1995), and can be summarized as follows: (i) it is sequence-specific; (ii) it results from a variable but substantial decrease in levels of mRNA; (iii) it affects only some

plants from the same transformation event; (iv) once established, gene silencing tends to be stable; and (v) it does not appear to result from permanent genetic modifications.

Newel et al. (1991) reported that only 25% of plants recovered from *Agrobacterium*-mediated transformation of potato, screened under severe antibiotic pressure, expressed coat protein from potato virus X at levels detectable by ELISA. In the same experiment, a construct carrying genes for the coat protein of two viruses simultaneously (potato viruses X and Y) produced only a proportion of plants that were able to express one or the other coat proteins, and a smaller proportion expressing both. The authors did not attempt to explain this phenomenon.

The literature also reports the importance of the ELISA conditions, which may indicate the need for optimization of some parameters in the ELISA procedures for detection of PSV-CP in red clover. Antigen preparation in our experiments was usually carried out by extracting plant sap with coating buffer, composed of sodium carbonate and sodium bicarbonate, pH 9.6, with no other additives. The addition of chelating agents, such as EDTA and NaDIECA, could be an alternative (Heuvers and Peters, 1989). Smith and Banttari (1987) contemplated the possibility that these compounds could stabilize virus nucleocapsid by reducing oxidized phenolic compounds, or could facilitate the release of virions from

cells. In our case, since we are dealing with coat protein alone, instead of whole viral particles, some of these effects may not be observed.

McLaughlin et al. (1984) used Tween 20 in all extraction buffer combinations tested in double antibody sandwich (DAS)-ELISA assays for detection of viruses infecting plant viruses. In the plate-trapped antigen indirect (PTA-I)-ELISA adopted in our experiments, however, the use of Tween 20 in the extraction buffer should be avoided (Garnsey and Cambra, 1991). Other steps in ELISA, such as incubation time, plant sap dilution, and number of washes, could also be considered.

High background in ELISA is a common problem, and has been observed in a variety of plant and animal systems (Trottier et al., 1992). In some cases, increased resolution without increasing the background has been observed (Henry and Francki,1992); for instance, improvement in the detection of potato leafroll virus (PLRV) was achieved by the addition of sodium diethyldithiocarbamate (NaDIECA) or EDTA to the sample buffer (Heuvel and Peters, 1989). Smith and Banttari (1987) discussed the possibility that these additives could stabilize virus nucleocapsid by reducing oxidized phenolic compounds, or could facilitate the release of virions from cells.

An assay on the effect of additives in sample-grinding buffer on detection of clover yellow vein virus (CYVV) and

white clover mosaic virus (WCMV) by ELISA showed significant differences between several additives on the viral detection; in general, addition of 0.05% Tween-20 and 20 µM NaDIECA to PBS buffer gave the best results (McLaughlin et al., 1984). However, in some cases, the absorbance of healthy plant extracts were higher than, or close to, the absorbance of infected plant extract, for example when Tween 20 was the sole additive in the extraction buffer for the detection of CYVV.

Background is influenced by various factors, mainly incubation time and components on the extraction buffer, as well as the use of blocking agents to avoid non-specific binding. Other factors include choice of microplate, antigen immobilization, optimal dilution of sera, and kinetics of the enzymatic reaction (Trottier et al., 1992).

Henry and Francki (1992) optimized extraction and detection parameters for barley yellow dwarf luteovirus (BYDV), a phloem-restricted virus that occurs in very low concentrations in grasses. Overnight incubation of samples at 25 °C in 100 µM citrate buffer, pH 6.0, or 100 µM phosphate buffer, pH 7.0, prior to testing increased resolution without increasing the background. Celluclast added to the incubation buffer was also beneficial in increasing resolution.

Cross-absorption of serum with sap from healthy citrus plants before use was required to yield a positive reaction

for detection of citrus tristeza virus with some polyclonal antibodies (Rocha-Pena et al., 1991). In the same system, several blocking agents were tested in western blotting procedures (3% BSA, 3% gelatin, 0.5% non-fat dry milk, or 5% Triton X-100), and all gave adequate white background on nitrocellulose membranes; gelatin, however, gave the best contrast between the green/purple color for healthy and infected plants, respectively. Improvement in the detection of potato leafroll virus (PLRV) was achieved by the addition of sodium diethyldithiocarbamate (NaDIECA) or EDTA to the sample buffer. This significantly increased the A_{405} values of infected plants, while reducing the background signals (Heuvel and Peters, 1989).

The presence of 20 μM Mg^{2+} resulted in a two-fold increase in nonspecific background in an indirect ELISA for serodiagnosis of *Actinobacillus pleuropneumoniae*. In this animal system, no differences in antigen immobilization were found by using buffers of various compositions and pH levels (Trottier et al., 1992). In indirect ELISA tests, Schloter et al. (1992) observed that a test with fluorescein was not suitable for the quantitation of *Azospirillum brasiliense* in soil, because the background fluorescence of soil components interfered. Cho et al. (1991), also using an animal system, were able to reduce background associated with bovine viral diarrhea (BVD) virus by solubilizing the virus with the

detergent MEGA-10 (decanoyl-N-methylglucamide), followed by removal of hydrophobic proteins with Triton X-100.

A study on the effect of additives in sample-grinding buffer on detection of clover yellow vein virus (CYVV) and white clover mosaic virus (WCMV) by ELISA showed significant differences between several additives on viral detection. In general, addition of 0.05% Tween-20 and 20 µM NaDIECA to PBS buffer gave the best results (McLaughlin et al., 1984). These authors also tested the effect of sap from healthy white and red clover in the extraction buffer, however there nas no influence of the health saps on the optical density for the control plates. The A_{400} for red clover sap ranged from 0.03 to 0.10, contrasted with the A_{400} for infected plants, which presented values as high as 1.29. However, in some cases, the optical density of healthy plant extracts were higher than, or close to, the optical density of infected plant extract, for example when Tween 20 was the sole additive in the extraction buffer for the detection of CYVV (A_{400} of 0.09 and 0.10, respectively for buffer plus Tween 20 without and with the addition of red clover sap).

Considering this information, we also analyzed some variables in connection with the ELISA assay protocol for this system. There was an almost linear relation between sap concentration and A_{405}, higher sap concentration giving higher readings; however, even the lowest concentration tested (1:50 dilution, w/v) gave adequate readings when

sodium carbonate/bicarbonate coating buffer was used as the extraction agent.

The use of PBS as extraction buffer, or the addition of anti-oxidants to sodium carbonate/bicarbonate coating buffer had a marked effect on the ELISA results, reducing the A_{405} in all cases except for the two negative controls. The significance of this is two-fold. The lower values obtained with these alternatives to sodium carbonate/bicarbonate coating buffer used in the sap extraction mixture make the comparisons within each ELISA run more difficult, since many positive plants would present absorbance readings closer to the ones obtained with negative controls. In addition, because the background, or 'noise', is not reduced in the negative controls, the comparisons are even more difficult.

In practical terms, therefore, sap dilutions of 1:3 or 1:6 in coating buffer should give good results in red clover transgenic plants.

Table 5-1. Optical density at 600 nm (OD_{600}), recorded 12 and 24 hours after inoculation of the medium, for colonies of *Agrobacterium tumefaciens* strain EHA101(pMPSV4-43). Cells were made competent by the calcium chloride method, and transformed by adding pMPSV4-43 plasmid DNA, followed by freezing in liquid nitrogen and thawing at 37 °C. Cells were selected on solid YEP medium containing 100 μg mL^{-1} spectinomycin, 50 μg mL^{-1} kanamycin and 50 μg mL^{-1} streptomycin, and grown on liquid YEP medium with the same concentration of antibiotics.

Colony	Hours	
	12	24
	-------- OD_{600} ---------	
EHA101(pMPSV4-43)/1	0.371	0.511
EHA101(pMPSV4-43)/2	0.409	0.843
EHA101(pMPSV4-43)/3	0.008	0.262
EHA101(pMPSV4-43)/4	0.057	0.543
EHA101(pMPSV4-43)/4	0.015	0.263
EHA101(pMPSV4-43)/6	0.011	0.229
EHA101(pMPSV4-43)/7	0.001	0.112
EHA101(pMPSV4-43)/8	0.056	0.395
EHA101(pMPSV4-43)/9	0.717	1.081
EHA101(pMPSV4-43)/10	0.628	1.052
EHA101(pMPSV4-43)/11	0.707	1.045
EHA101(pMPSV4-43)/12	0.648	0.963
EHA101(pMPSV4-43)/13	0.628	0.994
EHA101(pMPSV4-43)/14	0.455	0.801
EHA101(pMPSV4-43)/15	0.594	0.946
EHA101(pMPSV4-43)/16	0.592	0.935
EHA101(pMPSV4-43)/17	0.612	0.976
EHA101(pMPSV4-43)/18	0.564	0.911
EHA101(pMPSV4-43)/19	0.447	0.551
EHA101(pMPSV4-43)/20	0.688	1.107
EHA101(pMPSV4-43)/21	0.440	0.540
EHA101(pMPSV4-43)/22	0.477	0.799
EHA101(pMPSV4-43)/23	0.411	0.523
EHA101(pMPSV4-43)/24	0.398	0.487
EHA101(pMPSV4-43)/25	0.694	1.148
EHA101(pMPSV4-43)/26	0.633	1.071
EHA101(pMPSV4-43)/27	0.637	1.088
EHA101(pMPSV4-43)/28	0.757	1.143
EHA101(pMPSV4-43)/29	0.485	0.654
EHA101(pMPSV4-43)/30	0.467	0.562
EHA101(pMPSV4-43)/31	0.375	0.560
EHA101(pMPSV4-43)/32	0.659	1.082
EHA101(pMPSV4-43)/33	0.484	0.852
EHA101-no antibiotics	0.427	0.564
EHA101-with antibiotics	0.025	0.047

Table 5-2. Absorbance values at 405 nm (A_{405}) for several plants of red clover (*Trifolium pratense*) exposed to *Agrobacterium tumefaciens* strain Z707(pMPSV4-43), and tested for transformation on several occasions using plate-trapped antigen indirect enzyme-linked immunosorbent assay (PTA-I-ELISA). **Methods**: red clover petiole parts were cocultivated with *A. tumefaciens* for 48 hours, then transferred to B5 callus induction medium with 300 μg mL^{-1} carbenicillin and 50 μg mL^{-1} kanamycin. ELISA was performed on regenerated plants maintained in a green-house, and on *Vigna sinensis* (L.) Savi infected with PSV as control, using antiserum against peanut stunt virus coat protein (PSV-CP) or neomycin phosphotransferase type II protein (NPTII). Values shown are the lowest and the highest for each category.

Test Date	Anti-serum	No. plants	Control positive	Control negative	Test plant
			---------- A_{405} ----------		
08-94	PSV-CP	32	0.765-0.856	0.112-0.210	0.077-0.170
09-94	PSV-CP	32	0.301-0.335	0.001-0.040	0.007-0.052
10-94	NPTII	23	0.092-0.344	0.035-0.113	0.004-0.166
10-94	PSV-CP	80	2.614-2.999	0.856-1.039	0.934-1.076
01-95	PSV-CP	16	1.175-2.097	0.113-0.489	0.211-0.526
08-95	PSV-CP	30	2.323-2.999	0.850-1.200	0.126-0.415

Table 5-3. Mean absorbance values at 405 nm (A_{405}) for several plants of red clover (*Trifolium pratense*) exposed to *Agrobacterium tumefaciens* strain Z707(pMPSV4-43) and tested for transformation on two occasions using plate-trapped antigen indirect enzyme-linked immunosorbent assay (PTA-I-ELISA). **Methods**: red clover petiole parts were cocultivated with *A. tumefaciens* for 48 hours, then transferred to B5 callus induction medium with 300 µg mL^{-1} carbenicillin and 50 µg mL^{-1} kanamycin. ELISA was performed on regenerated plants, using antiserum against peanut stunt virus coat protein (PSV-CP). Values shown are the average for nine replicates.

Test Date	Anti-serum	No. plants	Control positive†	Control negative††	Test plant†††
			---------- A_{405} ----------		
09-95	PSV-CP	3	2.585	0.199(C)	1.138(L) 1.200(H)
10-95	PSV-CP	25	2.104	0.609(C) 1.245(G)	0.620(L) 1.332(H)

† Control positive was *Vigna sinensis* infected with PSV.
†† Control negative plants were maintained in a greenhouse (G) or in plastic tissue culture containers in the laboratory (C).
††† All test plants were maintained in growth chambers in the laboratory; values shown are averages for the plants presenting the lowest (L) and highest readings (H).

Table 5-4. Mean squares from the analysis of variance for absorbance at 405 nm (A_{405}) from a plate-trapped antigen indirect enzyme-linked immunosorbent assay (PTA-I-ELISA) for several plants of red clover abd tobacco transformed with *Agrobacterium tumefaciens* Z707(pMPSV4-43), EHA101(pMPSV4-43) and EHA101(pMON9793).

Source of variation	Degrees of freedom	A_{405}
Plant	18	4.419 **
Block	3	0.300 **
Error	206	0.008

** F test significant at the 1% level.

Table 5-5. Absorbance at 405 nm (A_{405}) for several plants of tobacco (*Nicotiana tabacum*) transformed with *Agrobacterium tumefaciens* strains EHA101(pMPSV4-43) and EHA101(pMON9793), and red clover (*Trifolium pratense*) transformed with strain C58-Z707(pMPSV4-43). **Methods**: tobacco leaf discs or red clover leaf petioles were cocultivated with *A. tumefaciens* for 48 hours, then transferred to medium with 300 $\mu g\ mL^{-1}$ carbenicillin, plus 100 $\mu g\ mL^{-1}$ kanamycin (tobacco) or 50 $\mu g\ mL^{-1}$ (red clover). Plate-trapped antigen indirect enzyme-linked immunosorbent assay (PTA-I-ELISA) was performed on regenerated plants, using antiserum against peanut stunt cucumovirus coat protein.

	Absorbance
	--- A_{405} ---
Red clover	
Plant #19 (N80)	1.200b†
Plant #30 (N80)	1.169b
Plant #27 (N50)	1.130b
Control negative	0.199g
Tobacco	
Plant #11 (TMPSV-20-11-2)	0.861c
Plant #9 (TMPSV-20-30-2)	0.836c
Plant #13 (TMPSV-11-06-1)	0.799c
Plant #15 (TMPSV-20-15-1)	0.574d
Plant #7 (TMPSV-11-13-1)	0.476e
Plant #6 (TMPSV-20-11-1)	0.463e
Plant #5 (TMPSV-20-11-4)	0.447e
Plant #1 (TMPSV-11-10-1)	0.354f
Control negative #1	0.184g
Plant #3 (TMPSV-20-01-1)	0.174gh
Control negative #2	0.173gh
Plant #4 (TMPSV-11-06-2)	0.094hi
Plant #65 (TMON9793-06-6)	0.083i
Controls	
Vigna sinensis PSV-infected	2.585a
Vigna sinensis healthy	0.126ghi

† Means with the same letter within columns do not differ at the 5% level (Duncan's multiple range test).

Table 5-6. Mean square values from the analysis of variance for absorbance at 405 nm (A_{405}) from a plate-trapped antigen indirect enzyme-linked immunosorbent assay (PTA-I-ELISA) for several plants of red clover transformed with *Agrobacterium tumefaciens* EHA101(pMPSV4-43), and C58-Z707(pMPSV4-43). The assay used different extraction buffers and plant sap dilutions.

Source of variation	Degrees of freedom	Mean square
Plant (P)	5	9.203 **
Extraction buffer (E)	3	5.555 **
Sap dilution (D)	4	2.251 **
E*P	15	0.943 **
E*D	12	0.133 **
Error	200	0.029

** F test significant at the 1% level.

Table 5-7. Absorbance at 405 nm (A_{405}) for several plants of red clover (*Trifolium pratense* L.) transformed with *Agrobacterium tumefaciens* strain C58-Z707(pMPSV4-43) and tested with several extraction buffers and plant sap dilutions. **Methods**: red clover leaf petioles were cocultivated with *A. tumefaciens* for 48 hours, then transferred to medium with 300 µg mL^{-1} carbenicillin and 50 µg mL^{-1} kanamycin. Plate-trapped antigen indirect enzyme-linked immunosorbent assay (PTA-I-ELISA) was performed on regenerated plants, using antiserum against peanut stunt cucumovirus coat protein.

	Absorbance
	---A_{405} ---
Species/plant	
Vigna sinensis PSV-infected	1.449a†
Red clover #17 (N50P3)	0.690b
Red clover #18 (N80)	0.502c
Red clover #20 (N80)	0.422d
Red clover control negative #1	0.162e
Red clover control negative #2	0.156e
Extraction buffer	
Sodium carbonate/bicarbonate (NaCB)	1.015a
PBS pH 7.4	0.472b
NaCB + 2% PVP	0.397c
NaCB + 200 µM NaDIECA	0.370c
Sap dilution	
1:3	0.864a
1:6	0.698b
1:12	0.496c
1:25	0.440c
1:50	0.320d

† Means with the same letter within each variable (species/plant, extraction buffer, and sap dilution) do not differ at the 5% level (Duncan's multiple range test).

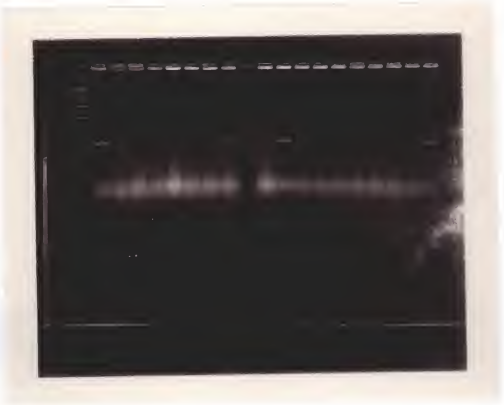

Fig. 5-1. The presence of the DNA segment containing the peanut stunt virus coat protein (*psv-cp*) gene in *Agrobacterium tumefaciens* colonies transformed with the plasmid pMPSV4-43, demonstrated by polymerase chain reaction (PCR). Lane 1, 1 kb DNA ladder; lanes 2, 12, and 20, strain Z707(pMPSV4-43); lanes 3 through 9, and 13 through 19, colonies from strain EHA101 transformed with the above plasmid (colonies 1, 11, 15, 20, 28, 29, and 30, respectively); lane 10, all PCR reagents except template DNA; lane 11, strain EHA101 non-transformed. Methods: a single bacterial colony was boiled in 100 µL of water for 1 minute, and mixed with the PCR reagents. Primers for the *psv-cp* gene were used. The PCR conditions were: DNA denaturing at 94 °C for 1 min and 30 seconds, primers annealing at 60 °C for 1 min and 30 seconds, and elongation at 72 °C for 2 min and 30 sec. The cycle was repeated 30 times. PCR products were run in a 1.2% agarose gel at 90 volts for 2 hours, and stained with ethidium bromide.

Fig. 5-2. The presence of the DNA segment containing the *npt*II gene in tobacco and red clover plants which were subjected to transformation by *Agrobacterium*-mediated DNA transfer, demonstrated by polymerase chain reaction (PCR). Lanes 10, 20, and 30, 1 kb DNA ladder; lanes 1-9, 11, 23, 24, and 29, putative tobacco transformed with plasmid pMPSV4-43; lanes 12-19, 21, and 22, putative red clover transformed with plasmid pMPSV4-43; lanes 25, 26, and 28, tobacco, red clover and citrus, respectively, transformed with *npt*II gene with plasmid pMON9793. The following PCR conditions were used: DNA denaturation at 94 °C for 1 minute, primers annealing at 60 °C for 1 min, and chain elongation at 72 °C for 3 min, for 40 cycles, followed by final chain elongation at 72 °C for 5 min. Genomic DNA was used at rates of 300 ng per 50 µL reaction. Amplification products were electrophoresed on 2.0% agarose gel at 90 volts for 2 hours and stained with ethidium bromide.

Fig. 5-3. The presence of the DNA segment containing the peanut stunt virus coat protein (*psv-cp*) gene in tobacco and red clover plants which were subjected to transformation by *Agrobacterium*-mediated DNA transfer, demonstrated by polymerase chain reaction (PCR). Lanes 10, 20, and 30, 1 kb DNA ladder; lanes 1-6, 9, 11, 13-18, 28 and 29, putative tobacco transformed with plasmid pMPSV4-43; lane 6 is accession TPSV20-11-2, which presented high A_{405} value in the ELISA assay, and lane 11 is TPSV20-11-3, both originated from the same leaf disc; lanes 22-29, putative red clover transformed with plasmid pMPSV4-43; lane 7, control tobacco plant, non-transformed; lane 12, all PCR reagents except template DNA. The following PCR conditions were used: DNA denaturation at 94 °C for 1 min, primers annealing at 60 °C for 1 min, and chain elongation at 72 °C for 3 min, for 40 cycles, followed by final chain elongation at 72 °C for 5 min. Genomic DNA was used at rates of 300 ng per 50 µL reaction. Amplification products were electrophoresed on 1.2% agarose gel at 90 volts for 2 hours and stained with ethidium bromide.

CHAPTER 6
CONCLUDING REMARKS

The main objective of the studies discussed in the preceding chapters was to obtain transgenic red clover plants containing the *psv-cp* gene, whose expression would hopefully confer resistance to peanut stunt virus. Previous transformation attempts with the *npt*II gene in red clover were successful, although differences were observed in the ease of transformation for combinations of plant genotypes and bacterial strains. In our research, we initially used the vector construct Z707(pMPSV4-43) in combination with several plants of the NEWRC red clover germplasm. Plant regeneration in this species was very slow, but after approximately 12 months ca. 200 plants were obtained that had been regenerated under the standard protocol for red clover, i.e., the use of 50 $\mu g\ mL^{-1}$ kanamycin in the regeneration media, to select for transformants. Since the plasmid pMPSV4-43 did not contain reporter genes, we had to rely on ELISA and PCR tests to determine transformation. Many attempts to demonstrate the presence of the inserted DNA and the expression of the protein in the putative transformed plants were done, and this was eventually achieved.

We also carried out additional projects, that could optimize the transformation and selection conditions for red clover. We examined the effects of geneticin, an antibiotic that had successfuly replaced kanamycin in some experiments reported in the literature. We found that geneticin would be a desirable alternative to kanamycin for selection of transgenic red clover plants. Although more experimentation is needed on this area, probably using callus regeneration instead of seedling growth to assess the effectiveness of geneticin, this antibiotic was found to be resistant to deactivation by heat, which can greatly simplify the tissue culture procedures, since this would allow its addition to the medium prior to autoclaving.

We also investigated the use of acetosyringone, a phenolic compound known for its ability to induce the *vir* operon in *A. tumefaciens*. The addition of this compound could potentially increase the rate of plant transformation. In the studies reported here, the use of acetosyringone in the medium decreased the rate of bacterial growth, but increased the bacterial virulence, as noted by the more abundant callus formation in explants that were transformed with *A. tumefaciens* which had been conditioned in medium containing acetosyringone. Some differences were also noted in the response of *Agrobacterium* strains to acetosyringone, and, most importantly, the response of red clover explants to *Agrobacterium* strains. Some strains were able to induce

larger calli in selective medium, indicating that more efficient transformation had been achieved.

We devoted considerable effort dealing with *Agrobacterium* transformation, since we were interested in introducing the plasmid pMPSV4-43 into the strain EHA101. Not reported in the previous chapters are also attempts to transform EHA101 with two additional plasmids, pGAoCP and pGABYG, which contain the sense and anti-sense sequences for bean yellow mosaic virus coat protein, respectively. Cell were transformed on several occasions by direct DNA uptake and selected in medium containing tetracycline, resistance to which is encoded by those plasmids. The protocol used called for 3 $\mu g\ mL^{-1}$ tetracycline; however when this concentration was used, a number of colonies developed but none was transformed. Other attempts with up to 50 $\mu g\ mL^{-1}$ tetracycline resulted, in all cases, in non-transformed colonies. On the other hand, transformation of *Agrobacterium* with the plasmid pMPSV4-43 was successful, and a number of colonies were transformed; however, *E. coli* transformation with this plasmid was never achieved.

Once the construct EHA101(pMPSV4-43) was obtained, it was used to transform red clover and tobacco explants. We also used the construct EHA101(pMON9793), which contains the scorable reporter gene *gus*, as control. Tobacco plants were obtained approximately four months after transformation, but red clover, as stated previously, regenerated very slowly,

and no plants large enough to conduct PCR or ELISA tests on, have yet been recovered.

Attempts to demonstrate the transformed nature of the recovered red clover and tobacco plants were successful. A_{405} readings from ELISA for some of the putative transformed plants were above the threshold value. Also, DNA amplification through PCR showed the expected bands. However, the techniques used were not as repeatable as anticipated. For PCR, for example, some false negatives were obtained, but optimozation of reaction conditions, particularly DNA concentration, improved the results. On the other hand, no false positives were obtained from non-transformed plants.

To conclude, the experiments conducted, analyzed and discussed in this research were able to provide an insight into the development of more efficient protocols for red clover transformation, which will certainly facilitate future work in this area. The use of more specific protocols should improve the probability of obtaining transformed red clover plants with virus resistance which can be utilized in the improvement of this species.

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BIOGRAPHICAL SKETCH

Fernando Adami Tcacenco was born on January 7, 1954, in Caxias do Sul, Brazil. He entered the field of agronomy quite by chance, when a school friend encouraged him to apply for the qualifying exam to enter the Joao Simplicio de Carvalho Technical Agricultural School, in Viamao, Brazil. At the conclusion of his high school, he matriculated at the Federal University of Rio Grande do Sul, in Porto Alegre, Brazil, where he completed the Bachelor of Science degree in agronomy.

Upon graduation, he joined the Institute for Agricultural Research of the State of Santa Catarina, in Brazil, where he had the opportunity to work at the Headquarters and at two different research stations, in the areas of natural grassland, as well as exotic pasture plants research.

He received his master's degree in 1981 from the University of Birmingham, England, in conservation and utilization of plant genetic resources, under the auspices of the German Agency for International Cooperation.

In 1992, he was accepted at the University of Florida's Agronomy Department for his Ph. D. in plant breeding and genetics.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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